Conservation genetics of the species complex *Cochlearia officinalis* L. s.l. in Britain

Estelle Gill

PhD thesis
The University of Edinburgh
2007

Declaration

I hereby declare that this thesis is composed of work carried out by myself, the undersigned, unless otherwise acknowledged, and that this thesis is of my own composition. This thesis has not in whole or in part been previously presented for any other degree or professional qualification.

Estelle Gill

September 2007

Acknowledgements

I have been lucky enough to have three excellent supervisors, who are not only good scientists, but also very nice people. So I would like to thank Pete Hollingsworth and Richard Ennos for their continued optimistic support, patient explanations of population genetic theory and for our two entertaining, if unsuccessful, trips to Skye looking for a very elusive *Cochlearia* population. I would also like to thank Tim Rich, for his enthusiasm, the time he spent on *Cochlearia* field work with me and for the benefit of his impressive knowledge of plant taxonomy.

This PhD was funded by Scottish Natural Heritage as part of the Scottish Rare Plants Project, and supported by the Royal Botanic Gardens Edinburgh.

Thank-you to all the landowners, SNH area officers, reserve managers, rangers, wardens and vice county recorders for their kind permission to sample at various sites and information about *Cochlearia* distribution. A big thank-you to Heather McHaffie for accompanying me on fieldwork and for teaching me so much about the geology, history and natural history of Scotland and Northern England. I am grateful to the horticulture staff for taking care of the *Cochlearia* collection. Thanks to all those people who kindly collected and sent samples for the project. Thanks to Prof. Peter Wyse Jackson for sharing details of his work on *Cochlearia* with me

I would like to thank the lab ladies: Dr Michelle Hollingsworth, Alex Clarke and Dr Jane Squirrel for their help, advice and technical assistance in the lab.

Thank-you to the following people for their friendship, support, scientific discussion and/or pub time: Vimi Lomax, Dr Kath Evans, Camila Martinez, Dr Will Goodall-Copestake, Dr Chris Kettle, Dr Alan Forrest, Dr Daniela Schill, Dr Nazre Saleh, Dr Peter Wilkie, Dr Michael Möeller, Dr Sophie Neale, Haja Kader, Jin-Hyub Paik, Yoon-Wook Hyun, Dr Mark Hughes, Dr Barbara McKinder, Prof. Mary Gibby and Dr Graham French.

Finally, thank you to my family and to Pablo Fuentes-Utrilla for their support.

Abstract

The genus *Cochlearia* is a taxonomically complex genus with a circumpolar distribution. In common with many other post-glacial colonisers it exhibits complex patterns of morphological and ecological variation. The genus has been the subject of continued taxonomic controversy, especially within the species complex *C. officinalis* s.l. The focus of this study was to investigate whether the three rare putative endemic *Cochlearia officinalis* s.l. taxa in Britain: *C. micacea*, *C. officinalis* subsp. *scotica* and *C. atlantica* were sufficiently distinctive to warrant endemic species or taxon status at any rank. Furthermore, to make conservation recommendations for the species complex based on the outcome of this investigation. The patterns of differentiation in *Cochlearia* were studied to gain insight into the processes that have driven morphological and ecological diversification in the group.

The six putative taxa in *Cochlearia officinalis* s.l. were considered in this study: *C. officinalis* s.s., *C. officinalis* subsp. *scotica*, *C. pyrenaica* subsp. *pyrenaica*, *C. pyrenaica* subsp. *alpina*, *C. atlantica* and *C. micacea*. Samples of *C. danica*, a member of the wider genus *Cochlearia*, were also included for comparison. The samples were screened for variation in AFLP fragments, morphological characters and chloroplast haplotypes. This is the first study focussed on the British *Cochlearia* to use the amplified fragment length polymorphism (AFLP) technique. Many qualitative morphological characters differences between populations were maintained in cultivation under standard conditions. Variation in some quantitative morphological characters was significantly different between taxon groups. The morphological characters combined did not distinguish between taxonomic groups. Variation was found in samples from the uplands only. Although there were three chloroplast haplotypes all but 6 out of 96 samples had the same haplotype and the chloroplast was not taxonomically informative. The AFLP data did not vary significantly between taxonomic groups, ploidy levels, habitats or geographical regions. There was significant AFLP variation between populations. The morphological and ecological diversity present among populations of *Cochlearia officinalis* s.l. in Britain is most likely to result from local ecotypic differentiation. The variation in *Cochlearia officinalis* s.l. could not be divided satisfactorily into taxa of species rank and so specific conservation of taxa within the complex is not recommended. Instead the maintenance of *Cochlearia* diversity can be achieved by the continued protection of the habitats in which the ecotypes grow.

Contents

1. Conservation, taxonomic complexity and the genus *Cochlearia*.

1.1 Introduction

In 1992, the European Union Habitat and Species Directive (Council directive 92/43/EEC) set out a methodology for conserving European habitats, flora and fauna. The aim of the directive was 'to promote the maintenance of biodiversity', by identifying species or habitats under threat and implementing measures to conserve them. In Britain, species are conserved under this legislation using Biodiversity Action Plans or BAPs. Action plans are written for species under threat to promote the maintenance or recovery of the species concerned. This process hinges upon accurate taxonomic identification and recording of the species' and their distributions.

In many plant groups distinguishing between different taxa is straightforward. However, in others, species limits can be difficult to determine and the observed variation appears continuous (Stace 1997). This type of variation is typical of taxa that have recently diversified into new habitats. The North Atlantic region, including the British Isles, has been strongly affected by cycles of glaciation and glacial retreat. This has led to the recurrent elimination of organisms over large geographical areas followed by re-colonisation (Hewitt 2000, Abbott & Brochmann 2003). Glacial retreat creates many vacant niches promoting rapid diversification and, therefore, the development of taxonomically difficult species complexes.

A recent review by Brochmann *et al*. (2003) noted that amongst hardy-endemic plant taxa in the North Atlantic region, there is not a single sexual diploid species suggestive of long-term evolution. Instead, the endemic taxa are all associated with some mechanism or other that might promote rapid evolution such as chromosome number changes, breeding system changes or hybridisation. Groups with mechanisms that promote rapid evolution also tend to produce a range of morphologies and ecologies that defy simple classification (Hollingsworth 2003). Such groups create taxonomic challenges, but also provide an opportunity to investigate the mechanisms underlying the origins of new species.

These groups provide a useful model system for studying contemporary speciation and ecological diversification. Members of diversifying groups may also become the subject of

conservation interest because they rapidly produce endemic species. The importance of these taxa may be emphasised in the North Atlantic regions where there are only a small number of endemic species. Around 50% of the vascular plant species on the Biodiversity Action Plan Short-list are in taxonomically complex groups (French 2003). However, the incorporation of these closely related micro-endemic species into conservation programmes is difficult.

The species action planning process is, as the name suggests, species based. Taxonomic complexity has led to severe difficulties in employing species-based conservation approaches in some groups e.g. *Euphrasia* (French 2003); *Epipactis* (Squirrell *et al*. 2002) and *Cerastium* (Brochmann *et al.* 2004). There is no provision for dealing with these species complexes without selecting poorly defined 'rare' variants of complex groups as conservation targets. Inevitably, difficulties arise because these poorly defined groups cannot be identified and recorded with any confidence. The relative importance to biodiversity of parts of an interlinked species complex cannot be easily predicted; instead they must be treated as a whole. Conservation programs must aim to conserve the biodiversity of complex groups without becoming mired in taxonomic complexity.

The species complex *Cochlearia officinalis* sensu lato (s.l.) provides an excellent opportunity to study both evolutionary and ecological divergence and its impacts on conservation. This complex has produced three putative endemic taxa in the British Isles since the Pleistocene glaciation (Brochmann *et al.* 2003, Koch *et al.* 1998): *C. officinalis* subsp. *scotica* (Druce) Wyse-Jackson, *C. micacea* Marshall and *C. atlantica* Pobed. All three taxa are of conservation interest, but the basic requirement to clearly define units to be conserved cannot be met. This project investigates the evolutionary mechanisms underlying the taxonomic complexity in the *Cochlearia officinalis* s.l. complex in an attempt to refine approaches to conservation in this, and other similar groups.

1.2 The genus *Cochlearia*

Worldwide, the genus *Cochlearia* comprises approximately 30 species with a temperate to arctic-alpine distribution (Nordal & Laane 1990b). The genus is a member of the family Brassicaceae. There are two clearly defined sections of the genus *Cochlearia* found in Western Europe, section *Glaucocochlearia* O.E Schulz and section *Cochlearia* O.E. Schulz. Section *Glaucocochlearia* is found in South-West Europe and includes three species endemic to Spain and Portugal. Section *Cochlearia* is found widely across Europe and the

circumpolar region. The genus contains taxa with a range of different chromosome numbers, the base chromosome numbers of all taxa are either n = 6 and n = 7. The species within section *Cochlearia* are poorly defined (Koch *et al*. 1996, Koch *et al.* 1999) and different geographical regions have been subject to different taxonomic treatments. Pobedimova's treatment of the genus (1970, 1971) included all the European taxa, but has since been regarded as a highly 'splitting' approach and many of the taxonomic groups were never accepted or have been abandoned. The list that follows is taxonomically unstable, but serves as a guide to the range of variation among the European *Cochlearia.*

1.2.1 European inland taxa

The base chromosome number of the following taxa is 2n = 6. ***C. pyrenaica*** DC., is an ancestral diploid (2n =12) distributed across the uplands of Europe. There are two other diploids with restricted distributions: ***C. macrorrhiza*** (Schur) Pobed., a rare lowland species, restricted to a few populations between the Eastern Alps and the Carpathians (Kochjarová *et al.* 2006) and ***C. excelsa*** J. Zahlbr. ex Fritsch., a diploid of the Eastern Austrian alps (Kochjarová *et al.* 2006). There are also a series of hexaploids (2n = 36) with restricted distributions in the Carpathian mountains: ***C. polonica*** Fröhl., ***C. tatrae*** Borb. ***C. borzaeana*** (Coman & Nyár) Pobed.*,* (Koch 2002, Kochjarová *et al.* 2006) and ***C. barvarica*** Vogt., restricted to two areas in lowland Germany (Koch 2002).

1.2.2 European coastal taxa

Cochlearia anglica* L.** is an octoploid (2n = 48), also with a base chromosome number of n = 6, distributed from Northern France, around the British Isles to Southern Sweden. The coastal taxa that follow all have a base chromosome number of n = 7. ***Cochlearia officinalis s.s., the tetraploid 2n = 24, has a similar distribution, except that it extends further north to the Arctic Circle (Jalas *et al.* 1996). ***Cochlearia aestuaria*** (Lloyd) Heywood, an ancestral diploid (2n = 14) is restricted to the coast of Northern Spain and Southern France. The hexaploid ***C. danica* L.**, (2n = 42) which is found from Portugal up to Scandinavia was originally coastal, but spread to many inland sites soon after the first motorways were built. ***Cochlearia fenestrata* R.Br**., and ***C. groenlandica* L**. are both arctic diploids (2n = 14).

In Fennoscandia**,** four subspecies of *C. officinalis* have been described (Nordal & Stabbetorp 1990, Nordal & Laane 1996), which may be equivalent to taxa that are treated as species in other countries. These four subspecies are as follows: ***C. officinalis* L. subsp. *officinalis***, which is equivalent to *C. officinalis* s.s.; ***C. officinalis* subsp. *norvegica*** Nordal &

Stabbetorp (may be similar to some *C. atlantica* populations, which also grow on shingle beaches); ***C. officinalis* subsp. *anglica*** (L.) Alef., which may be equivalent to *C. anglica* sensu L., but Nordal & Laane (1990) applied this name to the populations of Southern Scandinavia only; ***C. officinalis* subsp. *integrifolia*** (Hartm.) Nordal and Stabbetorp. an upland taxon, that is possibly equivalent to the Scottish *C. micacea* Marshall (Nordal & Stabbetorp 1990).

1.3 Taxonomic complexity

The *Cochlearia officinalis* s.l. complex in Britain encompasses: *C. officinalis* s.s., *C. officinalis* subsp. *scotica*, *C. atlantica*, *C. micacea*, *C. pyrenaica* subsp. *pyrenaica*, *C. pyrenaica* subsp. *alpina* (Dalby 1991). The *Cochlearia officinalis* s.l. complex is highly polymorphic with variable ploidy levels and ecological preferences. Taxa within the complex exhibit high levels of environmental plasticity and are poorly morphologically differentiated from each other (Elkington 1984). Nordal & Stabbetorp (1990) stated that 'there is not a single quantitative character that can reliably distinguish between [the *Cochlearia* ecotypes and cytotypes]'.

1.3.1 Taxonomic treatments

The *C. officinalis* s.l. complex has eluded the attempts of taxonomists to explain the variation within it and frequent taxonomic revisions have not led to a consensus. There is such a high level of variation distributed between disjunct habitats that it is difficult to declare them as one entity. However, failure to find diagnostic, discontinuous features with which to subdivide the complex has left field botanists struggling to apply species names to populations. Table 1.1 summarises the major changes in British *Cochlearia* taxonomy and the methods that have been used to define taxa. The three species (*C. anglica*, *C. officinalis*, *C. danica*) initially described by Linnaeus (1754) are the only species that can be easily distinguished from each other. These three species have maintained their species status ever since - with the exception of Suanté's treatment (1955) where *C. anglica* was put as a subspecies of *C. officinalis* (Table 1.1). After Linnaeus' classifications, a series of new species were described splitting *C. officinalis* L. into five (*C. alpina, C. scotica, C. micacea, C. atlantica*, *C. officinalis s.s*), collectively known as the *C. officinalis* s.l. complex (Table 1.1). Later, Gill (1971a, 1971b) questioned the distinctiveness of *C. scotica* and *C. officinalis* s.s. Gill *et al.* (1978) also concluded that the three chromosome numbers discovered in

British upland populations corresponded to the three described taxa *C. pyrenaica* subsp. *pyrenaica*, *C. pyrenaica* subsp. *alpina* and *C. micacea*. Some minor taxonomic changes are not shown in Table 1.1, especially those where taxa oscillated between the rank of species and subspecies.

Author/ publication	Taxonomic methods	New species defined or changes
Species Plantarum (Linnaeus 1754)	C, M	*C. anglica* L. *C. danica* L. *C. officinalis* L.
Manual of English Botany (Babington 1843)	E, X, M,C	New inland taxon: *C. alpina* Wats.
Journal of Botany (Marshall 1892)	M	*C. groenlandica* of Scandinavia found in N. Scotland
Journal of Botany (Marshall 1894)	E, X, M, C	New inland taxon: *C. micacea* Marshall
Report for the Botanical Exchange club (Druce 1929)	E, M	New coastal taxon: *C. scotica* Druce (previously thought to be *C. groenlandica*)
Saunté (1955)	Cyt, X, M	Kept rank of *C. danica, C. officinalis* Moved all other species to subspecies of *C. officinalis*
Gill (1971a, b, 1973, 1976, 1978)	Cyt, X	*C. scotica* cytologically similar to *C. officinalis* s.s. An inland taxon new to Britain supported by Gill's research: *C. pyrenaica* (diploid).
Pobedimova (1969, 1970)	M, E	New coastal taxon: *C. atlantica*
Wyse-Jackson PS (1990)	M, E	*C. scotica* changed to *C. officinalis* subsp. *scotica*
Third Edition of the Flora of The British Isles (Clapham *et al.* 1981)	R	Defined the *C. officinalis* complex containing (*C. officinalis C. micacea, C. scotica, C. alpina*) as more closely related than *C. danica* and *C. anglica*
Watsonia (Dalby 1990)		*Cochlearia officinalis* subsp *alpina* changed to *Cochlearia pyrenaica* subsp. *alpina*
BSBI Crucifer Handbook (Dalby 1991).	M, E, R	First guide to accept *Cochlearia atlantica* Pobed. *C. scotica* Druce considered uncertain.

Table 1.1: A chronological table of the main taxonomic changes in the genus *Cochlearia* in Britain. The authors and their methods are displayed, along with the changes that they recommended. Key to taxonomic methods: C = cultivation studies, X = crossing experiments, M = morphological characters, E = ecological study, R = review of other taxonomic work, Cyt = chromosome counts or cytological work.

1.3.1.1 Nomenclature used in this thesis

The taxonomic basis for this thesis is provided by the account of the genus *Cochlearia* in the Botanical Society for the British Isles (BSBI) 'Crucifer Handbook' (Dalby 1991 - (see 1.3.1.2). This treatment was used because it provides a detailed examination of all putative taxa in Britain. The only modification to this treatment used here is that Wyse-Jackson's (1991) subspecies classification of *C. scotica* as *C. officinalis* subsp *scotica*. Dalby (1991) expressed some doubt about the existence of the *C. scotica*, but did not remove it's species rank. *Cochlearia* taxonomy remains controversial and the list shown in section 1.3.1.2 is not universally accepted.

1.3.1.2 List of taxon names used in this thesis

A species list for the genus *Cochlearia* in the British Isles as described in the BSBI 'Crucifer Handbook' (Dalby 1991, amended by Wyse-Jackson 1991).

C. officinalis s.l. complex

- *C. officinalis* L. *sensu stricto* (s.s.)
- *C. scotica* Druce. (=*C. officinalis* subsp. *scotica* (Druce) Wyse Jackson)
- *C. pyrenaica* DC subsp. *pyrenaica*
- *C. pyrenaica* DC subsp. *alpina* (Bab.) Dalby.
- *C. atlantica* Pobed.
- *C. micacea* E. S Marshall

- *C. danica* L.
- *C. anglica*. L.

1.4 Factors contributing to diversity

1.4.1 Glacial and post glacial history

The diploid species *Cochlearia pyrenaica* is thought to have survived in Britain at the height of the last glaciation. The *Cochlearia* macrofossils were found in Southern England, in deposits dating back 20 000 years, at the time when the glaciers were at their furthest extent (Godwin 1975 in Lang 1995, Godwin 1964). The current assemblage of British *Cochlearia* probably originated from these refugia, although this does not rule out later colonisers from elsewhere. The retreat of the glaciers left many vacant niches for plants to exploit, driving diversification (Hurka & Neuffer 1997). Range expansion and contraction of plant species in response to cycles of glaciation mean that plants have been repeatedly isolated in small

populations, followed by subsequent mixing during re-colonisation (Abbott & Brochmann 2003). This process can create taxa with complex, reticulate relationships.

1.4.2 Polyploidy

The low levels of neutral genetic divergence between the polyploid taxa of Northern Europe suggest that they arose around the time of re-colonisation in the late glacial period (Koch *et al.* 1998). The evolution of *Cochlearia* since this time has been characterised by the production of multiple autopolyploids and allopolyploids (Elkington 1984). Polyploidisation is a powerful evolutionary force; between 50-80% of dicotyledon plants are thought to be of polyploid origin (Soltis & Soltis 1995, Soltis *et al.* 2003). Genomic re-arrangements and changes in gene expression can result in considerable differences between the polyploid and its parental taxa/taxon. Song *et al.* (1995) found that rates of genomic evolution among experimental allopolyploids of three species of *Brassica* were much greater than that of the parental genomes. When compared with diploids; allopolyploids exhibit increased heterozygosity and allelic diversity, providing more genetic variation upon which natural selection can act (Soltis & Soltis 1993). Although the most dramatic changes are seen in allopolyploids, where two genomes are combined, autopolyploids can undergo functional diversification among duplicated genes (Blanc & Wolfe 2004). Polyploid (either allopolyploid or autopolyploid) plants have the potential for more rapid adaptation, faster divergence and greater flexibility in response to the environment than diploids (Brochmann & Elven 1992). The genus *Draba*, also in the Brassicaceae, is in many ways similar to *Cochlearia* with a range of polyploids of post glacial origin and a complex, poorly defined morphology (Brochmann 1992). Changes in ploidy level within *Draba* have been linked with changes in ecological tolerance e.g. competitiveness, salt tolerance (Brochmann 1992).

Chromosome number differences provide the strongest supporting evidence for the current taxonomy in *Cochlearia*. However, plants of the same ploidy level do not necessarily have a common origin (Soltis & Soltis 1995, Levin 2001). For example, molecular evidence indicates up to thirteen separate origins for *Draba* with the same ploidy number, with complex reticulation between them (Brochmann *et al.* 1992). Multiple origins of derived hybridgenous taxa have also been reported for another post glacial re-coloniser *Sorbus*, on the Scottish Island of Arran (Robertson *et al.* 2004), and also for *Senecio cambrensis* (Ashton & Abbott 1992) and *Arabis holboellii* (Sharbel & Mitchell-Olds 2001). Complex patterns of diversity may also occur where there have been multiple origins for same ploidal level (Brochmann *et al.* 1992, Abbott & Brochmann 2003, Ashton & Abbott 1992, Grundt *et*

al. 2004). If *Cochlearia officinalis* s.l. taxa of the same ploidal level have multiple origins these species may not be monophyletic groups. This may help to may explain the complex patterns of variation present in the complex.

Taxonomic species	Base number	Ploidy level	Chromosome number
C. pyrenaica subsp. *pyrenaica*	6	Diploid	12
C. pyrenaica subsp *alpina*	6	Tetraploid	24
C. officinalis s.s.	6	Tetraploid	24
C. officinalis subsp *scotica*	6	Tetraploid	24
C. micacea	6	Tetraploid + 2	26
C. anglica	6	Octoploid	48
C. danica	7	Hexaploid	42

Table 1.2: The base chromosome number, the ploidy level and chromosome numbers recorded for taxa in the genus *Cochlearia* in Britain (Gill 1965, 1971a, b, 1973, 1976)

Evolutionary relationships have been inferred (Elkington 1984) from the chromosome counts of Gill (1965, 1971, 1973, 1976). His theoretical pathway has been added to and modified by recent sequencing and RAPD analysis (Koch *et al.* 1996, Koch *et al.* 1999). An ancestral diploid, closely related to the modern *C. aestuaria* and *C. pyrenaica,* is thought to be the source of the modern *Cochlearia* assemblage. A chromosome-doubling (autopolyploid formation) is hypothesised to have led to the formation of *C. officinalis* s.l.(2n = 24) including *C. officinalis* subsp. *scotica* and a second doubling to have created *C. anglica* (2n = 48). *Cochlearia micacea* (2n = 26) was derived through aneuploidy from *C. officinalis.* The chromosome number and origins of *C. atlantica* are unknown; although morphologically it is closest to *C. officinalis* s.s.

1.4.3 Ecological diversity

Divergence in the *C. officinalis* s.l. complex has been primarily driven by ecological differentiation with limited morphological and genetic differentiation (Koch *et al.* 1996). The *C. officinalis* s.l. complex has diversified into a number of ecological niches in Britain. The abiotic coastal habitats which it inhabits are as follows: shingle and sand beaches, sand dunes, coastal grassland, saltmarsh and brackish marsh, bird cliffs. In the uplands, *C. officinalis* s.l. can be found in the following habitats: snow beds, base-rich ledges, springs, streams; habitats with high heavy metal content: such as serpentine debris and old mine workings (Nagy & Procter 1997, Nordal & Laane 1990, Nordal & Stabbetorp 1989, pers. obs. 2003, 2004).

The propensity for adaptation and diversification in the family Brassicaceae has been noted in a number of studies: in *Capsella bursa-pastoris* (Linde *et al.* 2001), in *Arabidopsis thaliana* (Griffith *et al.* 2004), and in *Cardamine flexuosa* (Lihová *et al.* 2006). Some differential adaptive responses have been found in *Cochlearia* that allow them to exploit varied habitats. For example, Nordal & Laane (1990) found adaptive differences between ecotypes in their response to day length. *Cochlearia* also show heritable adaptation to high nutrient levels. Very large, robust plants grow near manured bird nesting sites; at nutrient poor sites the plants are much smaller and do not respond to increased nutrients by growing larger (Nordal *et al.* 1986, Russell *et al.* 1940). Life history traits such as reproductive output and size varied significantly between wild populations of *C. barvarica* and *C. pyrenaica* in Barvaria, Germany (Abs 1999).

1.4.4 Breeding systems

Information gathered on breeding systems in *Cochlearia* is patchy, but suggests that *Cochlearia* are predominantly out-crossers with a mixture of self-compatible and self-incompatible populations. Nordal & Laane (1990) found a mixture of self-compatible and self-incompatible populations in Finnmark, Iceland and Svalbard. The self-incompatible plants had heavily scented flowers to attract pollinators, as do the coastal populations of *C. officinalis* s.s. and *C. anglica* in Britain. Pollinators are less frequent in upland situations (Billings 1974). Nonetheless, data collected for *C. barvarica* (Paschke *et al.* 2005) and for Austrian mountain taxa (Koch 2002) also suggest that they are obligate out-crossers. British upland plants often have low seed set, suggesting self-incompatibility (pers. obs. 2003 - 2004, Rich & Dalby 1996), although this has not been empirically tested.

1.4.5 Hybridisation and reticulate evolution

Cochlearia taxa are normally spatially reproductively isolated from each other as a result of divergence into different ecotypes. However, the flowering times of all the British species overlap and where different taxa grow in sympatry, hybridisation and introgression can occur (Fearne 1977, Dalby 1991, pers. obs. 2004). Of the five hybrid types recorded in Britain only *C. officinalis* x *C. danica* and *C. officinalis* x *C. anglica* (Stace 1975, 1997) occur in significant numbers.

Hybridisation between plants of different chromosome numbers can result in hybrid inviability, sterility or hybrid breakdown. Where progeny are fertile, the hybrid plants can stabilise in new niches, independent of parental populations, spawning self sustaining

lineages (Rieseberg 1995). Hybrids have novel gene combinations that allow new responses to habitats and environmental pressures. Hybridisation experiments have been used in *Cochlearia* to study reproductive boundaries and genomic incompatibilities (Beeby 1898, Saunte 1955, Gill 1973). Saunte (1955) stated that 'differences in ploidal level [in *Cochlearia*] do not prevent almost normal gene exchange'. Subsequent work showed that irregular meiosis occasionally occurred in *Cochlearia* crosses between different taxa (Gill 1971, 1976), but that there were no genetic sterility barriers between *C. officinalis*, *C. micacea* and *C. pyrenaica* (Gill 1973). All *Cochlearia* hybridised and producing offspring of variable fertility in experimental crosses (Saunte 1955, Gill 1973, Nordal & Laane 1990, Koch *et al.* 1996). The results of experimental crosses among *Cochlearia* taxa are shown in table 1.3.

Cross	Hybrid Pollen Fertility	F1 seed viability	Backcross fertility
C. pyrenaica* subsp *alpina* x *C. officinalis	77.9%	Set seed but are largely sterile	-
C. micacea* x *C. officinalis	95%	100%	-
C.officinalis* x *C. officinalis* subsp. *scotica	100%	100%	-
C. danica* x *C. officinalis	70%	High viability	High leading to introgression.
C. anglica* x *C. officinalis	100%	~ 100%	Lowered

Table 1.3: Results of natural and experimental hybridisation between *Cochlearia* species by various authors, showing hybrid pollen fertility, F1 seed viability and backcross fertility where data is available (Beeby 1898, Saunte 1955, Gill 1970, 1971, 1976, 1978, Ferne 1977).

The extent of past and present hybridisation in the genus *Cochlearia* is hard to quantify. Morphological data on hybrid populations can often be misleading. The phenotypic expression of genes encoding morphological characters is not consistent in hybrids so there can be discrepancies between genetic and morphological relationships (Brochmann 1992). Molecular studies in groups showing reticulate evolution often yield unclear phylogenetic results (Rieseberg & Soltis 1991). Nonetheless, the incongruence between Random Amplified Polymorphic DNA (RAPD) data and chloroplast data in *Cochlearia* across Europe indicates that the hybridisation between different chloroplast lineages has occurred (Koch *et al.* 1996).

1.5 Taxa in the genus *Cochlearia* in Britain

This section provides a brief description of all the taxa in the genus *Cochlearia* found in the British Isles. This thesis focuses on relationships within the *C. officinalis* s.l. complex (excluding *C. anglica* and *C. danica*), within which taxonomic complexity is a major problem. *C. danica* and *C. anglica* are, however, relevant because they can hybridize with members of the *C. officinalis* s.l. complex and hybrid populations are occasionally found (Stace 1975, Ferne 1977, pers. obs. 2004-2005). All descriptions summarized from Dalby (1991), with minor modifications based on personal observations (2004-2005).

1.5.1 *C. officinalis* s.l. complex (or *C. officinalis* agg.).

In cases of taxonomic uncertainty populations are often denoted simply with the complex or aggregate name *C. officinalis* (s.l or agg.). Overall, *Cochlearia officinalis* s.l. is very widespread around the British Isles. *Cochlearia officinalis* s.l. (Figure 1.2) has a predominantly coastal distribution, but there are also many inland records in Northern England, South-West England, West Wales and Western Scotland.

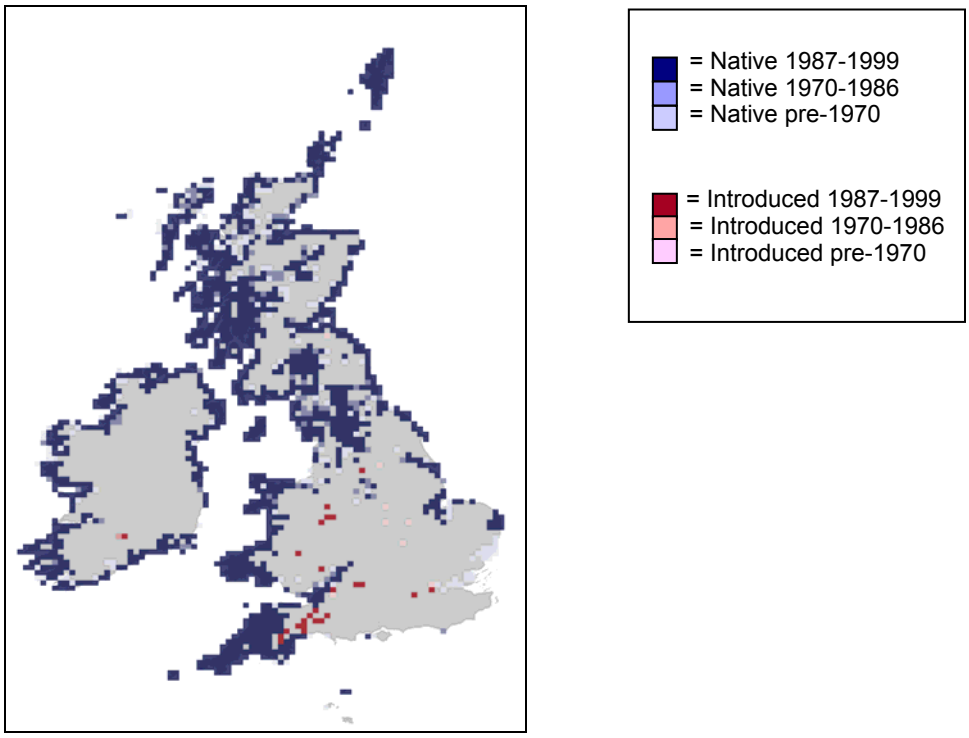

Figure 1.2: A distribution map of records for taxa in the *C. officinalis* s.l. complex in the British Isles. All distribution maps taken from Preston *et al.* (2002)

1.5.1.1 *Cochlearia officinalis* L. sensu stricto (s.s.)

C. officinalis s.s., or Common Scurvy-Grass (Figure 1.3), grows on nutrient rich cliffs, flushed coastal grass and in brackish marshes. It is associated with places that are damp year-round. *Cochlearia officinalis* s.s. reaches a height of 5-50cm with an erect or ascending growth form. The basal leaves are large and matt, with a cordate shaped base. Stem leaves are clasping, triangular ovate-oblong and entire, lobed or with blunt teeth. This species produces long flowering inflorescences. The petals are between 3-7mm long and white. Seed pods are rounded and 3-7mm x 2.5-6mm.

Figure 1.3: A specimen of *Cochlearia officinalis* s.s. growing at the base of cliffs at Port Gheiraha in the Outer Hebrides

C. officinalis s.s. was first described by Linnaeus in Species Plantarum (1753), it has a chromosome count of 2n = 24 and varies dramatically in size according to nutrient availability. When plants are found in intermediate habitats they are hard to distinguish from *C. scotica* or *C. atlantica*. *C. officinalis* s.s., refers to this specific taxon, whereas *C. officinalis* s.l. or *C. officinalis* agg. are often used to refer to the *C. officinalis* species complex in general. *C. officinalis* s.s. is widespread and has not been accorded conservation status; however, it could be a progenitor for the more localised variants of *Cochlearia*.

1.5.1.2 *C. officinalis* subsp. *scotica* (Druce) Wyse Jackson.

Cochlearia officinalis subsp. *scotica* or Scottish Scurvygrass (Figure 1.4) is found in a variety of nutrient poor, free-draining Scottish coastal habitats, including coastal grassland, rocks and shingle (Dalby 1991). It grows up to 5cm tall and normally has prostrate form. The basal leaves are less cordate than those of *C. officinalis* s.s. and much smaller (up to 2cm long). The stem leaves are sessile or with short stalks and do not clasp the stem. The inflorescences are short, often not emerging beyond the height of the basal leaves. The petals are white-purplish, squared and 3-4mm long, with a short claw. The seed pods are rounded and up to 3mm long.

Figure 1.4: A specimen of *C. officinalis* subsp. *scotica*, growing in rocks at Kerrara, near Oban

C. officinalis subsp. *scotica* was originally distinguished from *C. officinalis* s.s. on the basis of its smaller size, supposedly distinctive morphology and chromosome count of 2n = 14 (Gairdner 1939). Gill (1971, 1973) was unable to find any specimens of *C. officinalis* subsp. *scotica* with a chromosome count of 2n =14 (instead he found only 2n = 24) and regarded *C. officinalis* subsp. *scotica* as merely a morphological form of *Cochlearia officinalis* s.s. *C. scotica* was changed to *C. officinalis* subsp. *scotica* by Wyse-Jackson (1991) on the basis of poor morphological differentiation. Dalby (1991) believed *C. officinalis* subsp. *scotica* plants are *C. atlantica* or *C. officinalis* s.s. plants dwarfed by harsh growing conditions.

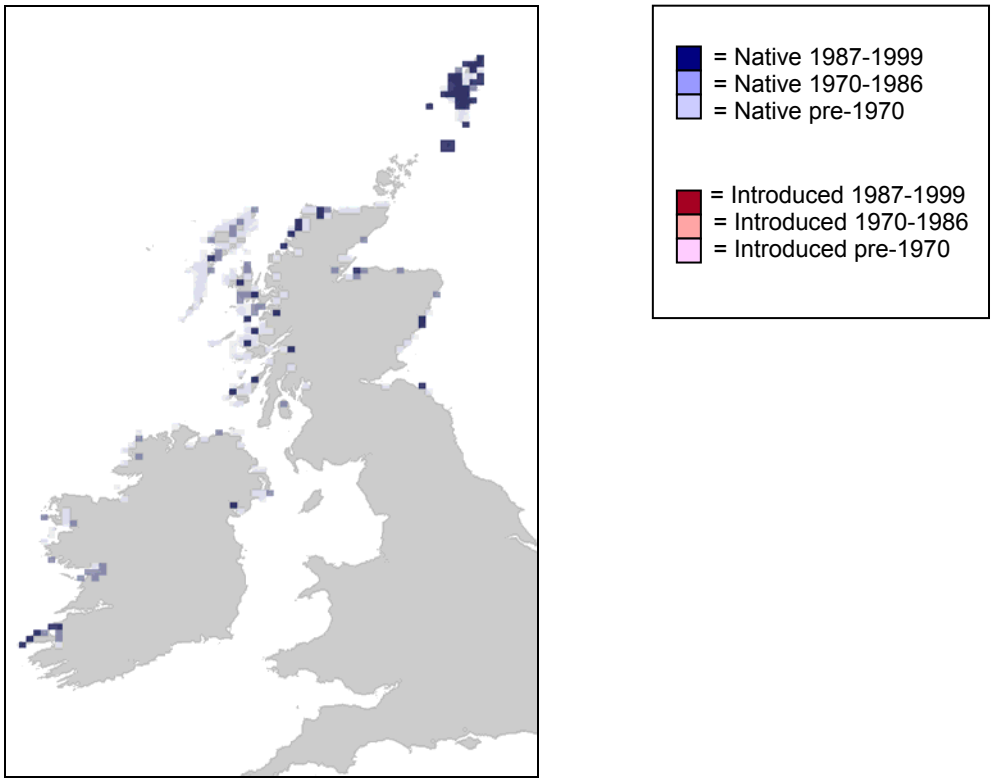

Figure 1.5: Distribution map of records for *C. officinalis* subsp. *scotica** records in the British Isles (Preston 2002).

C. officinalis subsp. *scotica* has been reported from the north and west coasts of Scotland, The Hebrides, Orkney, Shetland and Ireland (Figure 1.5 - Preston *et al.* 2002). The recording of *C. officinalis* subsp. *scotica* has reduced to virtually nil over the last few years, particularly since it lost species status. *C. officinalis* subsp. *scotica* is a Red Data Book species, being present in less than fifteen ten kilometre grid squares in Britain; and was selected as a priority action plan species. At the moment it is on the Red Data Book 'waiting list' pending taxonomic clarification. The taxonomic status of *C. officinalis* subsp. *scotica* must be confirmed before conservation resources are allocated to maintain its current range and populations.

1.5.1.3 *Cochlearia atlantica* Pobed.

This taxon grows on saltmarsh and on stony, sandy or silty sea-shores. It grows up to 20cm tall, with an erect or decumbent growth form. The basal leaves are up to 4cm long, normally with distinctive truncate based leaves and often with purplish colouring above or below. The stem leaves are sessile, entire or very slightly toothed. The petals are white and up to 5mm long. The seed pods are 2.5-4mm and rounded.

C. atlantica (Atlantic Scurvy-Grass) was first described by Pobedimova (1970) in her revision of *Cochlearia.* It has not been widely accepted and was not included in the 1993 edition of Flora Europaea (Tutin *et al.* 1993). Dalby (1991) gave the first detailed account of the species. The distribution of *C. atlantica* is unclear; Pobedimova (1970) and Dalby (1991) cited *C. atlantica* from only a few sites; however Rich (pers. com. 2007) considers the species to be more widely distributed around the Irish Sea. It as not been reported outside the British Isles and so it is a putative endemic. *C. atlantica* has been reported in Ireland, and on the west coast of Scotland, England and Wales, although no formal attempt as been made to map its distribution. *C. atlantica* is classified as 'data deficient' under IUCN criteria; this status reflects the taxonomic uncertainty surrounding this species.

1.5.1.4 *C. micacea* Marshall.

Cochlearia micacea (Mountain Scurvy-Grass) is an upland plant of wet, normally micaceous soils found between 600 and 1150m. *Cochlearia micacea* is biennial or perennial, with a dense compact from, growing to a maximum of 10cm tall. The basal leaves are up to 1cm long, orbicular or reniform, with a shallowly cordate or truncate base. Stem leaves are often toothed and clasping the stem. The petals are 2-4mm long and white. Fruits are up to 6 x 2mm long, and three times as long as wide, often asymmetrical with only slight veining or none at all.

C. micacea was first described by Marshall (1894) who demonstrated that the diagnostic characters remained consistent when in plants raised from seed *ex-situ*. *C. micacea* has a distinct chromosome number of 2n = 26 (Gill *et al*. 1978). Gill *et al.* (1978) was also satisfied that it was morphologically distinct. He described it as low growing with a perennial woody rootstock and with characters that suggested vegetative reproduction. After the discovery of the unique chromosome number (2n = 26) in *C. micacea* populations the taxon has maintained species rank in most Floras (Clapham *et al.* 1981, 1987, Stace 1997). Nordal & Stabbetorp (1990) suggested that *C. micacea* may be synonymous with *C.*

officinalis subsp *integrifolia* of Scandinavia and that accessory chromosomes are responsible for chromosome number counts of 2n = 26.

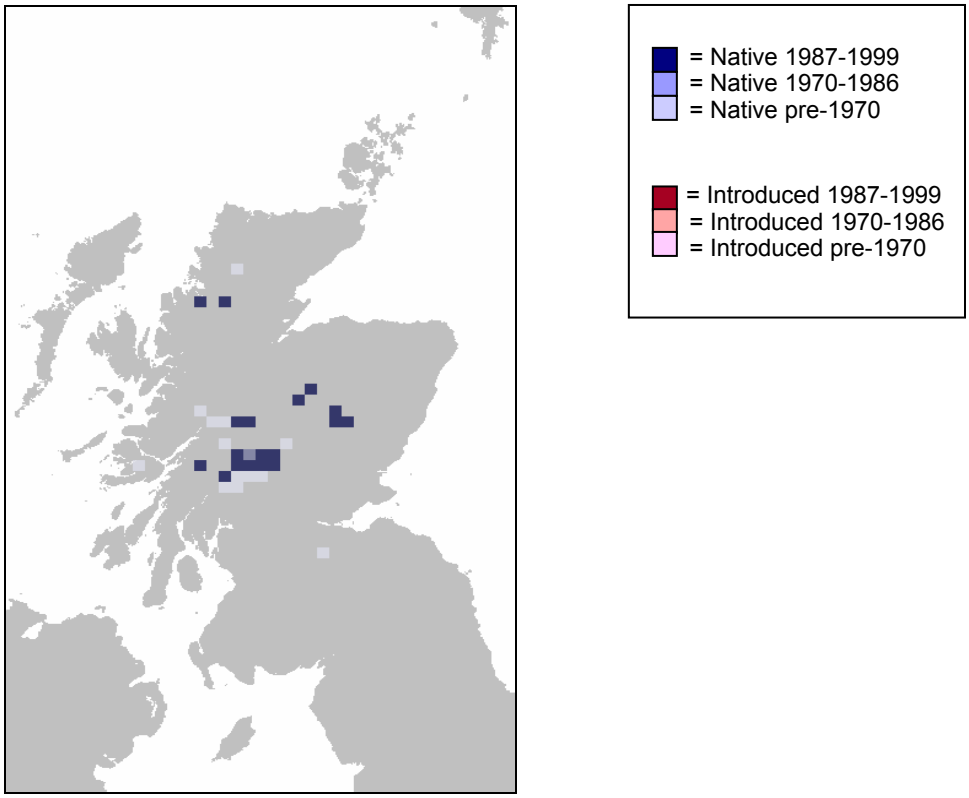

Figure 1.5: Distribution map of records for *C. micacea* records in the British Isles (Preston 2002).

C. micacea is locally common in the Breadalbanes (Dalby & Rich 1994). Reports of the species from other areas are uncertain (Figure 1.5). *C. micacea* is the subject of a UK Biodiversity Species Action Plan. The key requirement of the species action plan is to confirm the distinctiveness of *C. micacea*. Conservation objectives are simply to 'maintain all known populations in a viable state' (UK Biodiversity Steering Group 1995). Further conservation measures cannot be taken until *C. micacea* is confirmed as distinctive from *C. pyrenaica* subsp. *alpina*. Doubt over the distinctiveness of this species is reflected in the conservation treatment of the species:

'*C. micacea* [has] been removed from the priority list as it is highly likely that [it] is a subspecies of much commoner species' (Stirling priority species list 2002).

1.5.1.5 *Cochlearia pyrenaica* D.C

C. pyrenaica (Pyrenean Scurvy-Grass) in Britain has two subspecies *C. pyrenaica* subsp. *alpina* (tetraploid) and *C. pyrenaica* subsp. *pyrenaica* (diploid). The two cytotypes inhabit inland sites beside base rich streams or springs, often on substrates with high heavy metal content. They are biennial to perennial and up to 30cm tall. The basal leaves are up to 15mm long and reniform to heart-shaped with cordate to truncate bases. The leaf margins may be wavy or toothed. Upper stem leaves may also be toothed, often with a ragged appearance. The petals are up to 8mm long and white. The fruits are 3-5mm long and rounded, with reticulate veining.

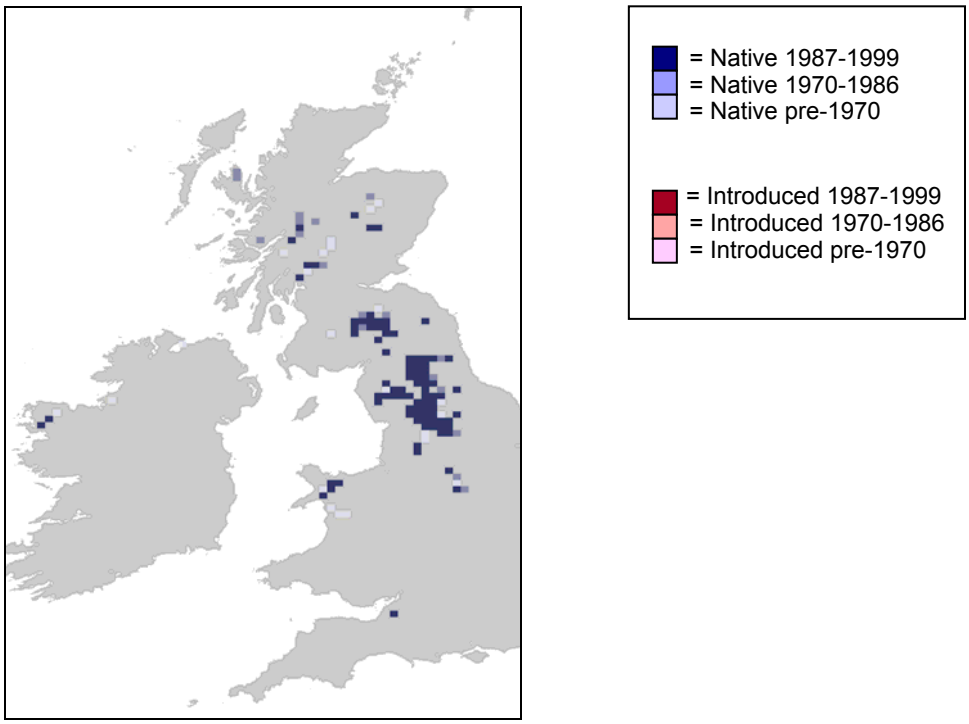

Fig 1.7: A distribution map of records for *C. pyrenaica* of both subspecies in the British Isles (Preston *et al.* 2002).

C. officinalis subsp. *alpina* was changed to *Cochlearia pyrenaica* subsp *alpina* is by Dalby (1990); the original name is often used. When *C. pyrenaica* subsp *alpina* was first defined, herbarium specimens from Scotland, Teesdale and York were included under the description, so this name applies to the diploid populations as do the names *C. pyrenaica* subsp. *pyrenaica* and *C. officinalis subsp. alpina* (Clapham, Tutin & Warberg 1952), leaving no name for the upland tetraploid of Scotland and Wales. The taxonomy needs to be formally resolved, but for the purposes of this thesis, *C. pyrenaica* subsp. *pyrenaica* is used to apply to the diploid populations of Northern England and Skye and *C. pyrenaica* subsp. *alpina* is

used to refer to the tetraploid populations of Wales and Scotland. Figure 1.7 shows the distribution of records for both species.

1.5.1.5.1 *C. pyrenaica* subsp *pyrenaica* Druce

This diploid taxon is ancestral to the European polyploids (Nordal *et a*l 1988). It is also found in the alpine areas of continental Europe.

1.5.1.5.2 *C. pyrenaica* subsp *alpina* (Bab) Dalby

This subspecies accounts for upland records of plants that are 2n = 24, which includes many of the Scottish populations not attributed to *C. micacea*. Upland tetraploids have not been found in continental Europe (Koch 2002). There may be distinct forms of *C. pyrenaica* subsp. *alpina* on serpentine debris (Rich 2003 *pers. com*.). Marshall initially named these plants *C. micacea,* but they have since been attributed to *C. pyrenaica* subsp. *alpina* (Dalby 1991).

1.5.2 *C. danica* L. and *C. anglica* L.

These taxa are outside the *C. officinalis* complex (Dalby 1991) and their distinctive morphological characters mean that they can be identified and delimited with more certainty than the taxa within the *C. officinalis* complex

1.5.2.1 *Cochlearia danica* L.

Cochlearia danica or Danish Scurvy-Grass (Figure 1.8) is an annual, pioneer species of sea-cliffs, sand dunes, coastal grassland and roadsides. *C. danica* is a winter annual that completes its life cycle before summer drought. It grows from between 3-10cm tall, but occasionally larger plants can be found in damp, nutrient rich sites. The basal leaves are orbicular-rounded triangular and die before seed set. The stem leaves are stalked and normally ivy-leaf shaped with lobes or teeth. The petals are 2.5-4.5mm long, often pink or purplish. The seed pods are pear shaped and 3-5mm long. The stalked stem leaves and pear shaped fruits consistently distinguish this taxon from other members of the genus.

Figure 1.8: A specimen of *C. danica*.

C. danica has consistently maintained its taxonomic rank since it was first described by Linnaeus (1753). It is hexaploid (2n = 42) with a base chromosome number of x = 7. It is largely self-pollinating and self-fertilizing (Gill 1976); despite this it readily hybridises with other British *Cochlearia* (Saunte 1955, Gill 1976). Before the 1970s *C. danica* was recorded only in coastal situations (Figure 1.9 – Preston *et al.* 2002); however, it has spread rapidly inland along motorways and is now found in urban areas (Scott 1985).

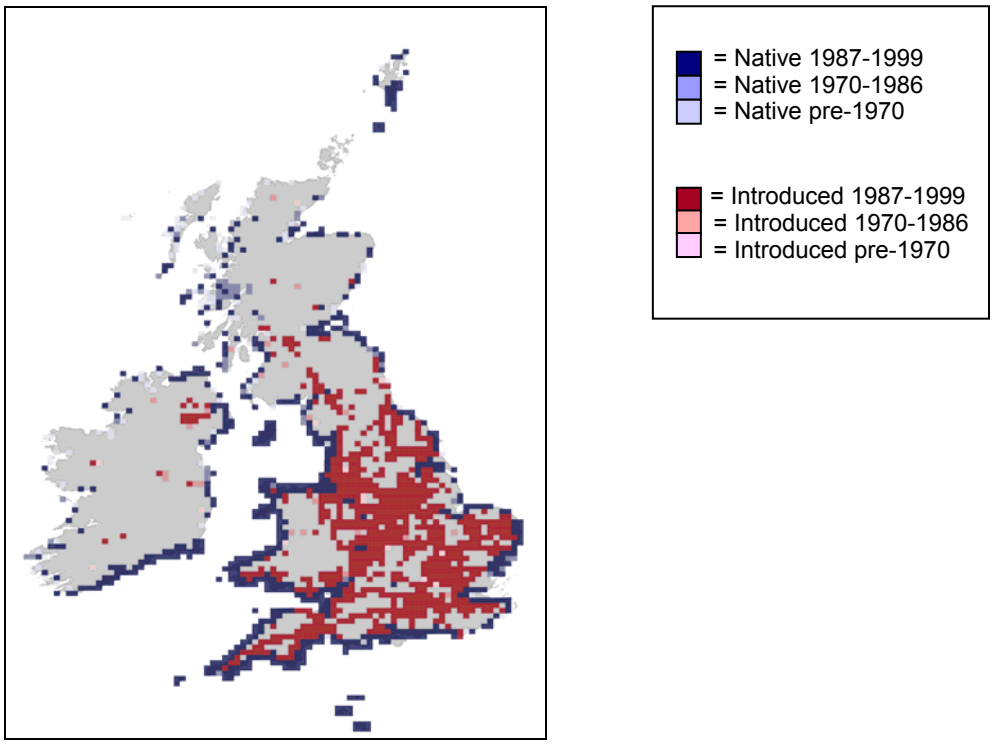

Fig 1.9: A distribution map of records for *C. danica* in the British Isles (Preston *et al.* 2002).

1.5.2.2 *C. anglica* L.

C. anglica or English Scurvy-Grass (Figure 1.10) is a biennial or perennial of soft mud found in estuaries or tidal rivers. *C. anglica* plants are 7-40cm tall. The leaves are lighter green than other British *Cochlearia* species. The basal leaves are elongated, ovate to obovate, with cuneate leaf bases. The stem leaves are also oblong. The petals are 5-10mm long and white. Seed pods, which are very distinctive from those of the other British members of the genus, are 3-4mm long and angustiseptate.

C. anglica has consistently maintained its taxonomic rank since it was first described by Linnaeus (1753), except in Scandinavia where it is classified as *C. officinalis* subsp. *anglica* (Nordal & Laane 1996). In Southern England and South Wales it is distinctive and easily recognised. Further north *C. anglica* intergrades with *C. atlantica* type plants. Figure 1.11 shows the recorded distribution of *C. anglica*, including some more doubtful records from Scotland.

Figure 1.10: A clump of *C. anglica*, at an estuarine site in France. (Photo by Erick Donnet)

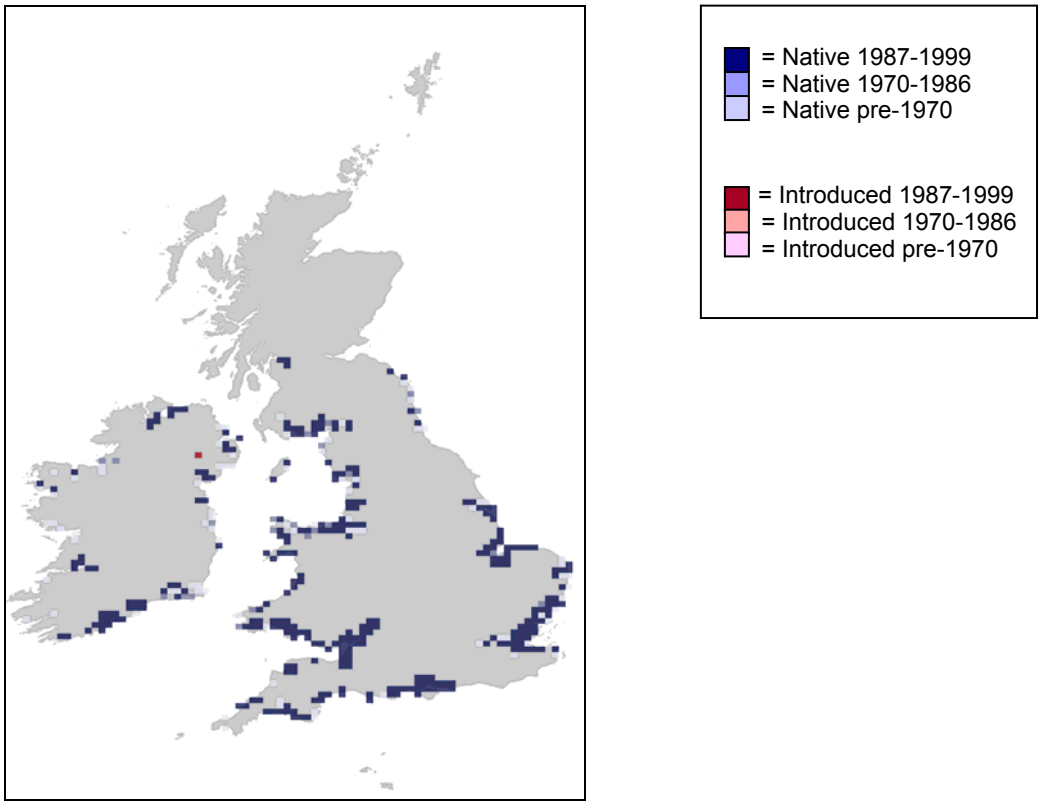

Figure 1.11: Distribution map of records for *C. anglica* in the British Isles (Preston *et al.* 2002)

1.6 Techniques to clarify taxonomic complexity in *Cochlearia*

In undertaking research on *Cochlearia,* it is important to build on existing data, but also to use new methods and collect new data to overcome some of the limitations of the previous studies.

1.6.1 Morphological taxonomy and morphometrics.

Traditionally, taxonomy has been based on morphological character differences between taxa. Species delimitation by macro-morphological characters is a pragmatic approach, because it defines the species and provides the characters with which to identify the species in the field. Various criticisms of sole reliance of morphological data have been made. Clusters of individuals with like-morphologies do not necessarily reflect evolutionary lineages. Morphological markers are often seen as inferior to molecular marker analysis because morphological variation is due to the expression of a small subset of genes (Hillis 1987)**.** Unless plants are delimited by using common garden experiments, then environmental factors may influence morphological characters.

The use of a morphometric approach to morphological taxonomy began with the numerical taxonomy work in the 1950's (Henderson 2006). This was an attempt to formalise morphological taxonomy and reduce subjectively. It also provided a reproducible method that could be used by different researchers for the same taxa. A large number of quantifiable morphological characters are measured. Then they are usually screened to choose the informative ones. Characters may also be weighted, depending on the importance the researcher gives them. Then a number of specimens are measured using the pre-defined characters. The data are then analysed statistically to look for clusters of like-morphologies. Selecting characters and then weighting them increases the subjectivity of the process. This type of data are normally analysed phenetically, therefore the character data is converted to frequencies of presence or absence. However, the results should be treated with caution as clusters of morphological types can appear that have no biological meaning, or which are not separated by characters (Goldstein *et al.* 2000).

Morphometrics have been used very effectively alongside molecular techniques to resolve taxonomy in complex plant groups. This approach has been useful for *Draba* (Scheen *et al.* 2002), *Cardamine pratensis* (Marhold *et al.* 1996) and *Festuca brachyphylla* (Fjellheim *et al.* 2001). Morphological characters can be added to phylogenetic trees to support groups and

to provide informative characters for identification (Scheen *et al.* 2002). If there is disagreement between the genetic and morphological characters then the researcher must decide which characters are more important or leave the groups unresolved.

1.6.1.1 What has been achieved with morphological markers in *Cochlearia*?

Identification and description of discrete morphological markers has been attempted many times to classify variation within *Cochlearia* (Marshall 1893, Clapham *et al*. 1952, Nordal & Laane 1990). Fruit and flower characters are favoured taxonomic characters because they are thought to be less environmentally influenced than characters such as leaf shape and growth form. Glasshouse growing experiments under standard conditions have been used to try and counter problems of environmental plasticity (Marshall 1893). Phytochambers have been used for morphological work on Scandinavian *Cochlearia* to eliminate environmentally mediated differences (Pegtel 1999, Nordal & Stabbetorp 1990, Nordal & Laane 1996).

Seed size, fruit shape and flower size proved discrete diagnostic characters among the *Cochlearia* of Svalbard, Iceland and Finnmark (Nordal & Laane 1990). Morphological variation was partitioned by ecotype and was maintained when growing conditions were standardised (Nordal & Laane 1990). Increase in leaf-base angles correlated with increasing chromosome number in continental European *Cochlearia* (Pegtel 1999). Taxa with different chromosome numbers also have distinctive seedlings, but these differences do not translate into discrete, unambiguous characters (Pegtel 1999). However, many of the character differences described above are related to chromosome number and are not useful for distinguishing British taxa, which do not differ in chromosome number (*C. officinalis* s.s., *C. atlantica*, *C. alpina* and *C. officinalis* subsp. *scotica* are all 2n = 24).

1.6.2 Molecular and cytological analysis

1.6.2.1 Genomes and techniques

Before embarking on studies of genetic variation using molecular markers, it is necessary to choose which techniques and which parts of the genome will highlight the genetic variation required to answer the question posed. Variation in the chloroplast genome is normally investigated by DNA sequencing one or more regions or using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). Electrophoretic techniques are used to screen for co-dominant isozyme markers derived from the nuclear genome. DNA sequencing is also used to amplify variable regions in the nuclear genome for example ITS or MatK. Techniques such as Amplified Fragment Length Polymorphism (AFLP) and

Random Amplified Polymorphic DNA (RAPD) amplify anonymous fragments that could be from the mitochondrial, chloroplast or nuclear genomes.

1.6.2.2 The attributes of the chloroplast genome compared with nuclear genome for molecular studies.

Markers from the chloroplast typically show more genetic-geographical structure than nuclear markers (Ennos *et al*. 1999). The chloroplast can be used to see historical relationships that have been erased by recombination in the nuclear genome. However, as all of the characters are inherited in a non-recombinant block, there is only limited statistical power and the chloroplast genome can be thought of as a single genetic locus (Ennos *et al*. 1999). Many natural processes can decouple nuclear variation and taxonomic characters from chloroplast lineages: contemporary gene flow (Lexer *et al.* 2007), cycles of hybridisation and differentiation (Guo *et al.* 2005) and recurrent polyploid formation (Brochmann *et al.* 2002). The amount of useful data gained depends largely on the amount of variation in the chloroplast. The chloroplast can show little or no polymorphism where species have undergone recent and rapid divergence (Koch *et al.* 1996, Guo *et al.* 2005, Després *et al.* 2002). Higher levels of polymorphism can often be detected in the nuclear genome, so markers utilising the nuclear genome can reveal more complex phylogeographical patterns than data from cpDNA loci alone (Magri *et al.* 2006).

1.6.2.3 Chloroplast sequencing and PCR-RFLP analysis

The chloroplast genome can be screened for polymorphisms by sequencing different regions. This is normally straightforward because universal primers have already been developed for conserved regions adjacent to variable regions; the variable regions can be amplified without the need to design new primers for specific study groups. Large amounts of chloroplast sequencing can be time consuming and expensive. An alternative is to use the polymerase chain reaction (PCR) with universal primers to amplify chloroplast regions. These amplified regions are cut with restriction enzymes using the Restriction Fragment Length Polymorphism (RFLP) method and the resulting fragments are run out on a gel. Changes at restriction sites will change the number of fragments and changes between restriction sites will give different sized fragments. Therefore, genetic differences between individuals can be identified by differences in banding patterns. RFLPs have been useful for clarification in complex groups, for example among apomictic *Rubus* species in Sweden, Denmark and Northern Germany (Kraft & Nybom 1995), and to clarify lineages between *Epipactis* taxa and populations in Britain (Squirrel *et al.* 2002).

1.6.2.4 Amplified Fragment Length Polymorphism analysis (AFLP)

The AFLP technique (Vos *et al.* 1995) produces a large number of uncharacterised genetic markers scattered across the genome (Mariette *et al.* 2002). AFLP analysis involves first a double digest of DNA with a frequent-cutting and rare-cutting restriction enzyme (Mueller & Wolfenbarger 1999). Then, an arbitrary subset of these fragments is amplified using two rounds of PCR. The fragments are labelled with florescent dyes then separated on an automatic sequencer. Single base changes will be detected if they are at restriction sites or adjacent areas. Deletions, insertions and rearrangements can alter presence/absence and size of restriction fragments.

This is a relatively cheap and simple method that produces a lot of markers quickly without the need for prior knowledge of the genome (Mariette 2002). The numbers of markers generated mean that AFLPs can detect very subtle genotypic differences. AFLPs provide markers for studies within populations, within species and between closely related species. Studies have shown that AFLPs are highly reproducible (Jones *et al.* 1997, Paun *et al.* 2006). Development time is required to screen for AFLP primers that provide a manageable number of polymorphic markers. AFLPs are dominant markers, so heterozygotes cannot be identified (Mueller & Wolfenbarger 1999). Theoretically, AFLPs amplify a fixed subset of the digested fragments each time because the primers and the high annealing temperatures only allow the amplification of target sequences. However, some non-specific binding can occur at the third base of a three-base specific primer (Vos *et al.* 1995). Experimental problems can also lower reproducibility. Partially digested DNA has been cited as the most common cause of reproducibility problems in AFLP analyses (Lin & Kuo 1995). Homoplasy is an acknowledged feature of AFLP datasets (Meudt & Clarke 2007). A study of fragment homology among AFLP fragments amplified from garlic (*Allium sativum*) found that 95% of same-sized fragments were identical and homologous (Ipek & Simon 2003). Some doubts have also been raised about whether AFLP markers are in fact randomly distributed throughout the genome. Breyne *et al.* (1999) found that different primer pairs produced different groupings in cluster analysis because they were preferentially amplifying markers from different parts of the genome.

The AFLP technique is being applied increasingly to delimit species and for phylogeny reconstruction in complex groups where ITS and chloroplast data do not have enough variation. This approach has been effective in *Trollius* (Després *et al.* 2003), *Soldenella* (Zhang *et al.* 2001) and *Androsace* (Schonswetter *et al.* 2003). However this approach did

not resolve relationships in *Minthostachys* (Schmidt-Lebuhn 2007). AFLPs have also been used to infer patterns of post glacial colonisation in *Minuartia biflora* and *Ranunculus pygmaeus* (Schonswetter *et al.* 2006b) and *Comastoma tenellum* (Schonswetter *et al.* 2004).

1.6.2.5 How have molecular markers and cytology contributed to taxonomic clarification so far in *Cochlearia*?

Chromosome number counts and cytological study provided a breakthrough in *Cochlearia* taxonomy (Saunté 1955, Gill 1965, 1971a, b, 1973, 1976, 1978). They supported many of the existing taxonomic groupings and allowed the formation of an evolutionary theory for the genus. Chromosome counts are of limited use to field botanists because they only apply with any certainty to specific sampled plants. The counts cannot be extrapolated to predict the chromosome counts of other populations with a similar morphology.

Intra-generic relationships in *Cochlearia* could not be resolved using ITS sequences or trnL chloroplast intron sequences (Koch 1999). Although chloroplast PCR-RFLPs on *Cochlearia* did yield informative results in widespread sampling across Europe (Koch *et al.* 1996). The levels of variation in the chloroplast among the European *Cochlearia* were extremely low. Among 89 populations taken from across Europe, ranging from Iceland to Estonia, only four mutations and six haplotypes were found when samples were screened with 25 restriction enzymes (Koch *et al.* 1996). The variation in chloroplast haplotypes did not correspond to morphological entities or ecotypes. RAPD markers were used in the same study and these were to some extent partitioned between ploidy levels and morphological types (Koch *et al.* 1996). An isozyme study by the same author also supported taxonomic grouping by ploidal level (Koch *et al.* 1998). This study also showed that the greater the number of chromosomes, the higher the allelic diversity, the exception being *C. pyrenaica* (diploid) which had the greatest allelic diversity (Koch *et al.* 1998).

The lack of resolution achieved using other markers makes *Cochlearia* an ideal candidate for a study using the AFLP technique. AFLPs have been used in two studies of montane *Cochlearia*, of the Alps and Eastern Europe (Koch *et al.* 2003, Kochjarová *et al.* 2006). The first study was used to define relationships between *C. macrorhizza* and the alpine and Eastern European montane *Cochlearia* populations. This study concluded that *C. macrorhizza* is a separate lineage and does not form a genetic bridge in between the montane populations. It also concluded that alpine *Cochlearia* taxa with n = 42 chromosome number had a single origin (Koch *et al.* 2003). In the second study the distinctiveness of Ukrainian

edge populations was defined. It was concluded that they were a distinct management unit. The study also confirmed the distinctiveness and endemic status of *C. borzeana* (Kochjarová *et al.* 2006). Although they found that most of the genetic variability was found within populations, there was differentiation between the Eastern European and Alpine samples (Cieslak *et al.* 2007). These studies show that AFLPs can be helpful in identifying distinctive populations in *Cochlearia* and clarifying the status of endemic species.

1.6.2.6 The use of molecular markers for post glacial colonisation studies.

The source populations for the British *Cochlearia* assemblage and the patterns of post glacial colonisation are unclear. Markers from the chloroplast genome and the nuclear genome can be used to make inferences about post glacial colonisation. The markers used need to evolve fast enough to accumulate changes in the post glacial period, but not so fast that homoplasy becomes a problem over the same timespan. However the amounts of informative variation recovered depend on the plant species in question and its history. The chloroplast region is commonly used for post glacial colonisation studies (e.g. Abbott *et al.* 2000, Guggisberg *et al.* 2006). The nuclear ITS region is also suitable for the post glacial time-span (Holderegger & Abbott 2003, Zhang *et al*. 2001). Isozyme polymorphisms have also been used as markers in many studies e.g. in *Draba* (Brochmann *et al*. 1992). More recently AFLPs have been used in post glacial colonisation studies to sample from across the nuclear and chloroplast genomes (Bronken *et al*. 2001), however they are normally combined with ITS or chloroplast data.

The genetic signatures resulting from post-glacial re-colonisation depend on many factors: the number of refugial source populations, the rate of colonisation, breeding systems within the species (Schaal *et al.* 1998). High genetic diversity is expected at a refugial site, with reduced diversity among populations of post glacial re-colonisers (Abbott & Brochmann 2003). This pattern can be disrupted by a population bottleneck (which leads to low genetic diversity) at a refugial site or by re-colonisers from different refugia meeting in a contact zone, creating high diversity in a colonised area. Plants that have re-colonised from more than one source refugia often have multiple within-species lineages (Comes & Kaderiet 2003, Sharbel *et al.* 2000). Weak or absent geographical structuring may be observed where there has been unstructured, recent colonisation from a single source population (Gaudeul *et al.* 2000, Después *et al.* 2002**,** Jørgensen & Mauricio 2004).

1.6.2.7 What have molecular markers revealed about post glacial population history in *Cochlearia*?

The low levels of variation in the chloroplast region make it very difficult to make inferences about population history in *Cochlearia* (Koch *et al.* 1996). There was, however, a pattern of successively lower allelic diversity in *C. officinalis* towards Northern Europe suggesting that step-wise re-colonisation with recurrent bottlenecks had occurred (Koch *et al.* 1998). Low divergence values between lineages are typical of taxa that have diversified post glacially (Hewitt 2000), as insufficient time has elapsed since glacial retreat for the formation of clearly separated endemic lineages. Lineages appear instead to have reticulate relationships (Koch *et al.* 1996).

1.6.3 Genetic data analysis

1.6.3.1 Options for analysis of AFLP data

In phylogenetic analyses, evolutionary models are used to deduce the most likely evolutionary explanation for contemporary patterns in character data. This kind of analysis has been attempted in a few studies using AFLPs (Angiolillo *et al.* 1999, Kardolus *et al.* 1998). The main drawback of attempting a phylogenetic approach with AFLPs is that evolutionary mechanisms causing fragment presence and absence are not well understood and no satisfactory evolutionary model has been made for use with AFLP data.

Most workers wanting to make inferences from AFLP data use phenetic methods based on similarity or distance measures. Analyses derived from presence or absence data converted to similarity or distance statistics e.g. Jaccard's similarity co-efficient, are less theoretically troublesome, than attempting phylogenetic analysis. Similarity statistics can then be used in cluster analysis, to construct phenograms, in principal co-ordinates (PCO) analysis or in analysis of molecular variance (AMOVA) analysis.

1.6.3.2 Principal co-ordinates analysis

PCO analyses are based on pairwise similarity of distance data. Similarity data are converted to values called Eigenvectors, which allow the individuals to be placed in multidimensional space in relation to each other. It is useful for data where there are lots of variables. This method can be applied to a wide variety of data types including morphological and genetic data. The first axis shows where the plane of greatest variation in the data is, and the second which is perpendicular to the first, shows the second greatest plane of variation, and so on. If the spatial pattern of the individuals corresponds to biologically meaningful variables, then the PCO plot can be used make hypotheses about the data. This method is useful for forming

hypotheses, but few firm conclusions can be drawn from it. Caution must be exercised in the interpretation of PCO plots or phenetic cluster analysis. Clusters can be heavily influenced by the number of loci scored in genetic data (Hollingsworth & Ennos 2004) and groups may separate without character differences (Goldstein *et al*. 2001).

1.6.3.3 The interpretation of phenetic data

Phenetic data analyses can only reveal contemporary similarity; they do not show historical patterns. Nonetheless, AFLP data are also increasingly used to make inferences about historical relationships, in *Carex atrofusca* (Schonswetter *et al*. 2006a), in *Primulaceae* (Zhang *et al*. 2001), and in cereal crops (Ozkan *et al.* 2002). Phenetic cluster diagrams produced from AFLPs are often concordant with ITS phylogenies, only with more resolution (Schonswetter *et al.* 2006a). This discovery has supported the use of AFLP data for the deduction of evolutionary relationships or to infer phylogeographical patterns. AFLP data have been used to make phylogeographic inferences in arctic-alpine groups such as *Androsaceae* (Schonswetter *et al.* 2003), *Gentianaceae* (Schonswetter *et al.* 2004), *Ranunculaceae* (Després *et al.* 2002). Genetic similarity could stem from historical gene flow, common origin or contemporary gene flow. Therefore, historical inferences should only be made with extreme caution.

1.6.3.4 AMOVA (Analysis of Molecular Variance)

AMOVA (Excoffier *et al.* 1992) is used to estimate the proportion of molecular variance accounted for by pre-defined groups. Pairwise distances are calculated and then the resulting matrix is used for an ANOVA based analysis. The AMOVA analysis can be used to test a pre-defined hierarchy, for example, variation between regions and variation between populations within regions. Estimates of population differentiation analogous to Fst can be derived from AMOVA analysis; this statistic is termed Φst. Φst is more robust than Fst when used with small or variable sample sizes. Compared with statistics based on the Hardy-Weinberg equilibrium (e.g. Fst, allelic diversity), AMOVA has few assumptions. However, neither measure can distinguish historical effects from contemporary relationships between populations (Schaal *et al.* 1998).

1.6.4 Approach to studying diversity in the *Cochlearia officinalis* s.l. complex in this thesis

Traditional morphological taxonomy has failed to resolve morphological complexity in *Cochlearia*. Characters that have proved useful in previous work on *Cochlearia* will be used

quantitatively to attempt to identify suites of characters for taxonomic groups. Markers obtained by PCR-RFLP will be used where there is sufficient variation in order to study historical relationships between taxa. AFLPs can be used to give an assessment of the partitioning of genetic diversity at a range of scales (national, regional and local) in British *Cochlearia*. The questions raised in this study in *Cochlearia* are at the interface of population genetics and phylogenetics and AFLPs are appropriate markers to use at this level.

1.6.5 Central questions tackled in this thesis:

1) Does the variation in AFLP markers, PCR-RFLP variation and morphological markers with the *C. officinalis* s.l. complex according to the existing taxonomic delimitations?

2) Specifically, are there distinct groupings of AFLP markers, PCR-RFLP variation and morphological markers that correspond to the three putative endemic species of conservation interest in Britain, *C officinalis* subsp. *scotica*, *C micacea* and *C. atlantica*?

2. An overview of genetic marker variation in *Cochlearia officinalis* s.l. in Britain.

Abstract

Patterns of genetic variation among the British *Cochlearia* have not previously been studied. An overview of genetic variation was needed before proceeding with more detailed studies. The usefulness and reliability of the AFLP technique for taxonomic clarification in *Cochlearia* was also assessed. Two samples were taken from a broad range of populations, and then screened for AFLP markers. The reproducibility of AFLP amplification and manual scoring were tested. No major problems were discovered with the quality or reproducibility of AFLP amplification and scoring. The genetic data was 'noisy' with low signal. There were no groupings of genetic similarity between individuals by chromosome number, taxon, regions or upland versus coastal habitats. Samples from the same populations were more similar to each other than samples from different populations, so variation was not entirely unstructured.

2.1 Introduction

No definitive taxonomy has been formulated for British or European *Cochlearia* using morphological markers. *Cochlearia officinalis* s.l. displays a wide range of morphologies and ecological preferences. However, the boundaries of these morphological and ecological types are difficult or impossible to define. Assigning putative groupings to test taxonomic hypotheses is challenging in *Cochlearia officinalis* s.l. because the taxonomy of the genus is so unsettled. No detailed studies of genetic variation have been undertaken among the British *Cochlearia*. Studies using isozymes (Koch *et al.* 1998), RAPDs and chloroplast haplotypes (Koch *et al.* 1996) have shown that *Cochlearia* has a complex, reticulate history in Europe. Chloroplast and ITS variation are commonly used to resolve phylogenies in complex groups (Bortiri *et al.* 2001, Holderegger & Abbott 2003). Previous studies have revealed very low variation in the ITS and chloroplast DNA sequences in the genus *Cochlearia* across Europe, thus markers from the ITS or the chloroplast genome are unlikely to be informative for a British study on a much smaller geographical scale.

2.1.1 Wide-scale surveys of AFLP variation in taxonomic investigations.

AFLP studies have made significant progress in defining groups among plant species where the taxonomy could not be resolved by other methods. Ideally, data derived from the nuclear

and chloroplast genome should be compared, but if this is not possible, AFLPs alone can provide useful information (Vijverberg *et al.* 2000, Després *et al.* 2002). AFLPs have been used to define groups in the recently diverged genus *Achilliea* (Guo *et al.* 2005) and *Trollius* (Després *et al.* 2002). The genus *Trollius*, like *Cochlearia*, has undergone an adaptive radiation since the Pleistocene glaciation but there has not been sufficient time for informative differences to appear in the ITS or chloroplast regions in these groups. In the genus *Soldanella,* AFLPs worked well at a fine taxonomic level, highlighting relationships between closely related taxa and indicating the geographical structure of divergence in the group (Zhang *et al.* 2001). AFLPs have also provided new and useful information on population structure for conservation in *Eryngium alpinum* (Gaudel *et al*. 2000), *Pedicularis palustris* (Schmidt & Jensen 2000) and *Oxytropis campestris* (Chung *et al*. 2004). AFLPs have been used to identify groups for conservation and resolve taxonomic problems in the *Cochlearia* of the Carpathian Mountains and Alps (Koch 2002, Kochjarová *et al.* 2007).

2.1.2 The use of AFLPs in polyploids

The number of fragments derived from AFLP analysis normally depends roughly on the size of the genome (Meudt & Clarke 2006). Therefore, it may be possible to see variation in fragment number between samples of different ploidy levels in *Cochlearia*. In *Cardamine pratensis* (Lihova *et al.* 2003) and *Euphrasia* (French 2003), there was a correlation between ploidal level and marker number. Although in *Veronica* polyploids (Martínez-Ortega *et al.* 2004), no connection was found between chromosome number and marker number. Fragment number is unlikely to be a sensitive enough measure to distinguish between *C. pyrenaica* subsp. *pyrenaica* (2n = 24) and *C. micacea* (2n = 26). It may be possible to distinguish between *C. pyrenaica* subsp. *pyrenaica* (2n = 12) and *C. officinalis* s.s (2n = 24).

2.1.3 AFLP quality control

AFLP fragments are uncharacterised; therefore, quality control measures are essential. DNA sequence data can be checked against published sequences. This allows the researcher to ensure that contaminant DNA has not been amplified. The individual AFLP fragments are too short to be checked against published data to ensure they came from the correct species, as sequences can be checked. The chances of amplifying contaminants are also heightened because the AFLP technique has two rounds of PCR. In addition, inconsistencies in manual checking of automatically scored data must be quantified. Homoplasy in AFLP datasets can be another source of inaccuracies in analysed results. Homoplasy is time consuming to

detect, but its occurrence in the dataset can be reduced simply, as described in section 2.1.3.1.

2.1.3.1 Elimination of small fragments

Homoplasy is a feature of AFLP datasets (Meudt & Clarke 2007) and it can lead to underestimates of differentiation. The question of homoplasy has been tackled in other studies by sequencing AFLP fragments purified from sequencing gels to check their homology (Rouppe van de Voort *et al*. 1997). This approach is very time consuming, because individual fragments must be sequenced. Homoplasy is highest among low molecular weight markers (Vekemans *et al*. 2002). In addition, low molecular weight fragments are often tightly packed together in size and difficult to score (pers. obs. 2006). Excluding low molecular weight fragments from the dataset is a straightforward way to reduce the problem of homoplasy and to eliminate fragments that may increase the rates of scoring error.

2.1.3.2 Duplicated fragments

Amplified fragments are of three types. The majority have an Mse restriction site at one end and an Eco restriction site at the other. There are also unlabelled Mse-Mse fragments and more rarely, labelled fragments with Eco sites at both ends. Mse-Eco fragments occur much more frequently than Eco-Eco fragments because Mse is a frequent cutter and Eco is a rare cutter, so there is a high probability of an Mse restriction site between each two Eco sites. If the same Eco primer is used for more than one of the primer pairs in the overall dataset, then the data from more than one primer pair could contain the same Eco-Eco fragments. These duplicated fragments need to be screened for because they could lead to over-estimation of similarity.

2.1.3.3 Assessments of reproducibility

AFLPs are said to be highly reproducible (Jones *et al.* 1997, Paun *et al.* 2006), however, under certain conditions, they may not be. Poorly digested DNA can cause inconsistencies in amplifications and therefore reproducibility problems (Lin & Kuo 1995). This can be tackled by running the digested DNA out on a gel, to check whether high molecular weight genomic DNA remains at the top of the lane. Duplicate control samples sound be added to each run to check for within-run reproducibility. Samples can be run on separate plates to check for between-plate reproducibility, or inter-plate comparisons can be avoided altogether.

Manual checking of automatically scored AFLP data can produce errors. Data from the automatic sequencer are scored using computer software, but still need to be checked manually. Error can occur during manual checking due to human inconsistency in scoring different individuals or datasets (Mueller & Wolfenbarger 1999). The same data can be re-scored to check reproducibly, however, it is almost inevitable that there will be some differences in scoring. The magnitude of scoring error can be quantified by calculating a percentage error rate (as described in 2.2.4.2).

2.1.4 Approach to investigating overall marker variation in British *Cochlearia*

This chapter outlines overall patterns of variation among the British *Cochlearia officinalis s.l.*, while the following chapters focus on specific areas of *Cochlearia* taxonomy. A wide-ranging sample scheme was used to identify groups and test taxonomic hypotheses. The reproducibility and reliability of the AFLP technique was also tested to ensure that subsequent conclusions are based on valid data. A duplicated sample was included to check for reproducibility between electropherograms. The results of one primer pair were scored twice and the percentage error induced by scoring was calculated. The central analytical approach was to test whether discontinuities in the data are congruent with the current taxonomy or with other known variables such as habitat, chromosome number and geography. A small number of samples were chosen from a wide range of populations to get an overview of variation within *Cochlearia*. Within-population similarity was tested against between population similarities. PCO analyses that identify uncharacterised clusters were combined with AMOVAs to test *a-priori* groupings.

2.1.5 Research questions

1) Is the AFLP approach robust in *Cochlearia*? Specifically:

a) When the same data are scored on two separate occasions, do the results change?

b) Is there any evidence for inter-dependence of data-sets if the same EcoR1 primers are used?

2) Are individuals within a population more closely related to each other than individuals from different populations?

3) Does AFLP variation contain groupings congruent with a) geographical, chromosome number or taxonomic group, or b) broad habitat groupings: upland or coast?

2.2 Method

2.2.1 Sample strategy

Samples were taken from thirty-four *Cochlearia* populations of seven taxa in England, Scotland and Wales. Two samples were analysed per population to gain an overview of variation. Herbarium specimens were collected for all populations at deposited in the Royal Botanic Garden Edinburgh Herbarium (E). Table 2.1 shows details of the taxa sampled, the population locations and the number of sampled populations. The location of the sites is shown in Figure 2.1. Due to the unsettled nature of *Cochlearia* taxonomy, definitive identifications of the samples were not always possible. Putative species identifications were based on reported chromosome counts for the population, morphological characters and habitat. The samples represented a wide range of ecological and morphological types. *Cochlearia* are difficult to classify, so where possible the individuals from the type locality were sampled, which are almost certainly the target species from the type description. Ben Lawers is the type location for *C. micacea*; Loch Linnhe is the type location for *C. atlantica,* and Tain is the type location for *C. officinalis* subsp. *scotica.*

Putative species	Region	Ploidy level (putative)	Site name and vice county	Grid reference	Description
C. officinalis s.s.	N England	24	Heysham Head, Lancashire	SD/407.612	Coastal sandstone cliffs
C. officinalis s.s.	N. Wales	24	Porth Colmon, Caernarvonshire	SH/194.342	On low cliffs and slipway, coastal
C. officinalis s.s.	E. Scotland	24	Crammond Island, Mid Lothian	NT/200.784	Rocks by the sea
C. officinalis s.s.	N W Scotland	24	Port Gheiraha 1, Outer Hebrides	NB/336.497	Coastal flushed bird-cliff
C. officinalis s.s.	N.W. Scotland	24	Ramasaig, North Ebudes	NG/159.436	Coastal rocks and shingle
C. officinalis s.s.	S. Wales & S.W England	24	Sand Bay, North Somerset	ST/322.630	West facing limestone cliffs coastal
C. pyrenaica subsp. *alpina*	N. Wales	24	Crib y Ddysgl, Caernarvonshire	SH/605.555	Base-rich upland flushed crags
C. pyrenaica subsp *alpina*	E.Scotland	24	Corrie an't-Sneachda, Easterness	NH/996.032	Scree filled flush, granite, upland
C. pyrenaica subsp. *alpina*	S.W Scotland	24	Ben Nevis, Westerness	NN/161.721	Flushed gully, upland
C. pyrenaica subsp. *alpina*	E. Scotland	24	Lochnagar, South Aberdeenshire	NO/246.785	Flushed gully, upland
C. pyrenaica subsp. *alpina*	E. Scotland	24	Cairnwell, Angus	NO/127.781	Mixed rock & limestone, stream gully, upland
C. pyrenaica subsp. *alpina*	Mid-Scotland	24	Meall nan Gabhar, Mid Perthshire	NN/234.724	Base rich crags, constant water, upland
C. pyrenaica subsp. *alpina*	E. Scotland	24	Little Kilrannoch, Angus	NO/218.772	Dry, serpentine gravel, upland
C. pyrenaica subsp. *pyrenaica*	N. England	12	Gordale Scar, Mid-West Yorkshire	SD/914.639	Limestone gravel, beside stream, upland

C. pyrenaica subsp. *pyrenaica*	N. England	12 or 24	Cawbank Spring, Cronkley Fell, North West Yorkshire	NY/856.279	Base-rich upland spring,
C. micacea	Mid-Scotland	26	Beinn Heasgarnich, Mid Perthshire	NN/429.379	Base-rich ledges, cliff base, upland
C. micacea	Mid-Scotland	26	Ben Lawers, Mid-Perthshire	NN/626.407	Wet flushed patches in grass, upland
C. micacea	Mid-Scotland	26	Ben Lawers2, Mid-Perthshire	NN/633.412	Micaceous, flushed ledges, upland
C. micacea	Mid-Scotland	26	Meall nan Tarmachan, Mid-Perth	NN/592.408	Crags below cliffs, base rich, upland
C. micacea	Mid-Scotland	26	Ben an Dothaidh, Main Argyll	NN/326 411	Micaceous shaded gully, upland
C. micacea	Mid-Scotland	26	Ben Lui, Mid Perthshire	NN/ 265 274	Flushed ledges, upland
C. officinalis subsp *scotica*	S.W. Scotland	24	Kerrara, Main Argyll	NM/802.266	Heavily gazed headland, coastal
C. officinalis subsp *scotica*	N.W. Scotland	24	Port Gheiraha 2, Outer Hebrides	NB/355.499	Bare rocks, short grass, coastal
C. officinalis subsp *scotica*	E. Scotland	24	Chanonary Point, East Ross	NH/747 556	Shingle beach, coastal
C. officinalis subsp *scotica*	E. Scotland	24	Tain, East Ross	NH/794.973	Mouth of tidal river, saltmarsh, coastal
C. atlantica	S.W. Scotland	24	Fort William, Loch Linnhe, Westerness	NN/087.764	Shingle beach and sea wall, coastal
C. atlantica	S.W. Scotland	24	North Oban, Main Argyll	NM/862 329	Among rocks on beach, coastal
C. atlantica	E.Scotland	24	Brora, East Sutherland	NC/908 089	Mouth of tidal river, saltmarsh, coastal
C. atlantica	N. Wales	24	Dyfi Estuary, Cardiganshire	SN/617.934	Saltmarsh, coastal
C. officinalis subsp. *scotica* OR *C. atlantica*	E. Scotland	24	Culbin Sands, Moray	NH/ 901. 577	Upper saltmarsh, coastal

C. pyrenaica subsp. alpina OR *C. officinalis*	N. W. Scotland	24	Stac Polly, West Ross	NC/111.103	Wet S-facing granite cliff, upland
C. officinalis subsp. *scotica* OR C. *atlantica*	N. W. Scotland	24	Inverpolly, West Ross	NC/137.906	Mixed saltmarsh and shingle, coastal
C. danica	S. Wales. & S. W. England	42	Llanwit Major, Glamorgan	SS/957.674	Sand dunes, coastal
Hybrids *C. danica* x *C.atlantica*?	N. Wales	?	Greenfield, Flintshire	SJ/199.779	tidal stream, nr dry sandy bank, coastal

Table 2.1: showing taxa, region, putative chromosome number, site name, grid reference, habitat type of sampled populations and number of samples used for widespread analysis of British *Cochlearia* The chromosome number is inferred from data gathered by other authors ((Gill 1965, 1971a, b, 1973, 1976)

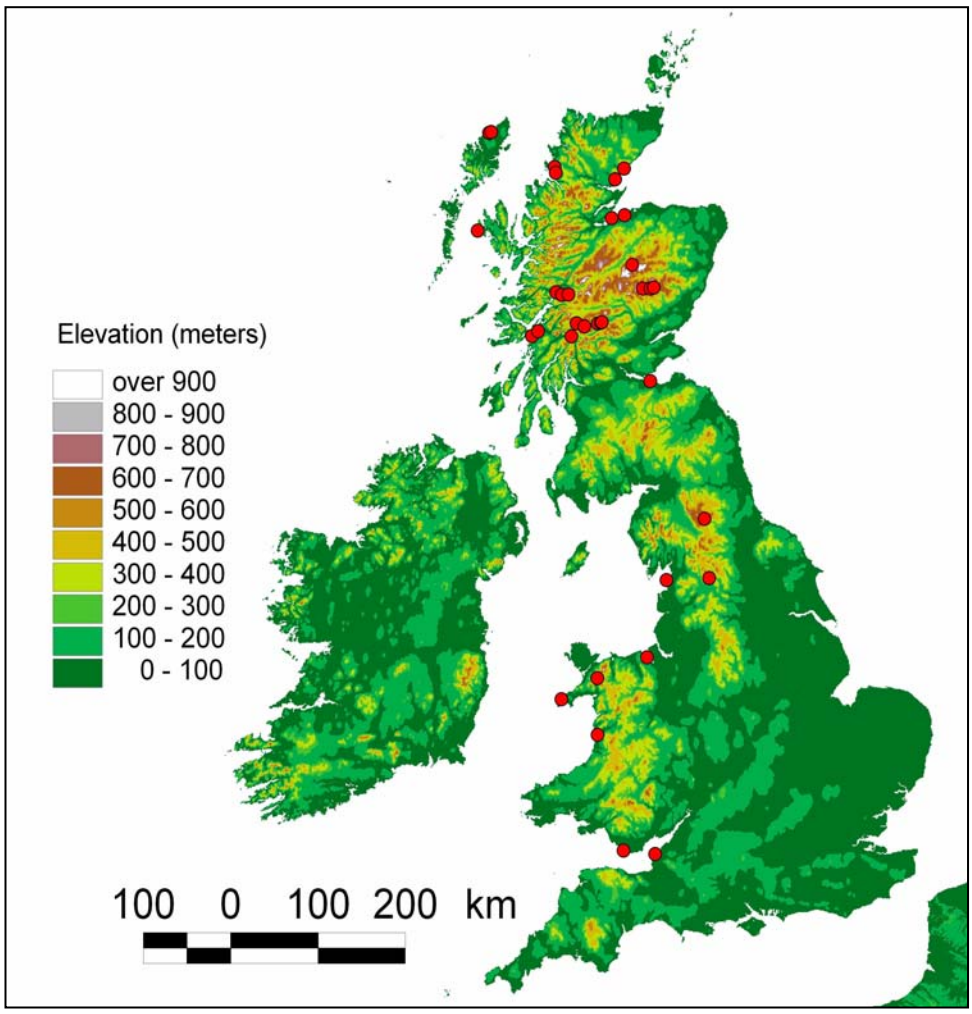

Figure 2.1: A map of Britain showing the locations of thirty-four populations of seven taxa sampled in order to gain an overview of genetic variation among British *Cochlearia*. The sampling focussed on populations of putative endemic taxa

2.2.2 DNA extraction

The DNA extraction was performed in two stages: a crude extract was made using the CTAB method. Then the DNA was purified using the Qiagen DNeasy Plant Mini Kit™.

The CTAB method was modified from the basic method by Doyle and Doyle (1987). Silica dried leaf material was put into a 1.5ml eppendorf with a tungsten bead. The tubes were loaded into a mixer mill and ground for 60 seconds, then rotated and ground again for another 20 seconds. Extraction buffer [1.4NaCl, 100mM Tris-HCl, pH 8.0], 20mM EDTA, 2% hexadecyltrimethylammonium bromide, 0.2% 2-mercaptoethanol, ~0.1X insoluble polyvinylpolypyrrolidine (PVPP) preheated to 65ºC was then added to the tubes. The mixture was then incubated at 65ºC for 30 minutes. 500µl of chloroform:isoamyl alcohol

(1:21) was added and the tubes were shaken on an orbital shaker for 20 minutes. Afterwards, the tubes were centrifuged for 10 minutes at 13,000 rpm. The supernatant was transferred to a new tube, and the chloroform extraction was repeated. Cold isopropanol at 2/3 the volume of the sample was added and the DNA was left to precipitate overnight. The precipitated DNA was pelleted by centrifugation for 10 minutes at 13,000 rpm. After removal of the supernatant, 1ml of wash buffer [76% ethanol, 10mM NH4Ac] was added and the tubes were left overnight again. The pellet was then spun down for 5 minutes at 13,000, the supernatant was removed and then the pellet was dried in the vacuum centrifuge for ~5 minutes. The pellet was then re-suspended in 100µl of distilled water. The quality and quantity of the extracted DNA was then assessed on 1.0% agarose gels.
DNA was then cleaned using plant mini-kit following the method described in The DNeasy® Plant Handbook (2006), with the following modifications: the method was started at step 7 and the optional step 10 was included.

2.2.3 AFLP method

AFLP analysis was used to generate markers from across the genome. The DNA was restriction digested with two enzymes EcoR1 and Mse1, then a two-stage amplification process followed, as described in Vos *et al*. (1995) with some modifications. AFLPs were processed in batches on 96 well plates. In order to fit all the samples on a 96 well plate only two samples per population were used in this dataset. The total number of samples analysed was 68. One sample per plate was duplicated to check for reproducibility within plates.

2.2.3.1 Restriction

Clean, high molecular weight DNA was incubated at 37ºC for 2 hours with restriction enzymes MseI & EcoRI and buffer; reagents as follows: 2µl 10x NEB2, 0.25µl EcoR1 (20u/µl), 0.1µl Mse1 (50u/µl), 0.5µl BSA (1mg/ml), 12.15µl dH_20. After DNA restriction, complete digestion was confirmed by running out 5µl of digested DNA on a 2% gel containing SYBR safe DNA visualisation stain, in 1% Tris-Borate-EDTA buffer, alongside a 1kb ladder (Bioline).

2.2.3.2 Making the adaptors

The following adaptors were prepared: Vos ECO Stock [5µM] forward adapter (5’ - CTC GTA GAC TGC GTA CC-3’), Vos ECO stock [5µM] reverse adapter (5’-AAT TGG TAC GCA GTC TAC-3’), Vos MSE Stock [50µM] forward adapter (5’-GAC GAT GAG TCC TGA G-3’), Vos Mse reverse adapter 5’-TAC TCA GGA CTC AT-3’) Stock [5µM]. The

Mse and Eco adapters were prepared for ligation separately by heating at 95ºC for 5min; they were then cooled to room temperature before use or storage. To make working solutions: Eco adapters 5µl [5µM] of forward and reverse ligation adaptors in 490µl of dH_2O; Mse adapters 10µl [50µM] each adapter in 80µl of dH_2O.

2.2.3.3 Ligation

15µl of the digestion mixture was transferred for ligation to the adapters. The ligation mixture per sample was as follows: 2µl 10x ligase buffer, 2.2µl EcoR1 adaptor, 1.1µl Mse1 adaptor, 0.025µl T4 ligase (400u/µl), 15µl restricted DNA (reagents all from New England Biolabs™). The reaction mixture was then incubated at room temperature for 3 hours. Te product of this reaction was diluted 1:10 to make the pre-amp template.

2.2.3.4 Pre-amplification

The pre-amplification (pre-amp) was performed using Mse1 and EcoR1 primers with the addition of a selective nucleotide on the 3' end. Amplification reactions used 3µl of diluted ligation product; 1.3µl of 10x PCR buffer (Bioline™); 0.5µl of $MgCl_2$ (50nm Bioline™); 1.3µl (dNTPs 2mM); 0.39µm Eco + 1 primer [10µm] TAGN™; 0.39µm Mse + 1 primer [10µm TAGN™], 0.1µl Taq polymerase (Bioline™) [5 units/µl], 6.02µm dH_20. The following PCR thermocycling profile was used: the initial denaturation was at 72ºC for 2 minutes, followed by 25 amplification cycles of: 94ºC for 20 seconds, 56ºC for 30 seconds, 72ºC for 2 minutes, then the final extension time of 60ºC for 30 minutes using an Applied Biosystems Geneamp PCR System 2700™. 8µl of the pre-amp product was run on a 2% gel to check that the amplifications had been successful. The pre-amp was then diluted 1:10 dilutions for use in the selective amplifications.

2.2.3.5 Selective Amplification

The primers used in the selective amplifications were optimised by trialling 13 Mse primers with 5 Eco primers in various combinations. The following species and populations (shown with the accession code and grid reference) were used to represent the range of British *Cochlearia* diversity for primer optimisation: *C. pyrenaica* subsp. *alpina*, Meall nan Gabhar MNG2 (NN/234.724) ; *C. officinalis* subsp. *scotica*, Uig, Uig5 (NB/050.329); *C. officinalis* s.s., Ramasaig RA2, (NG/159.436); *C. danica*, North Berwick NB10 (NT/559 852), *C. anglica*, Uphill BN5 (ST/309 582); *C. pyrenaica* subsp *alpina*, Crib y Ddysgl CD4 (SH/605.555), *C. micacea*, Ben Dothaidh, BD2, NN/326 411); *C. atlantica*, Brora BR1

(NC/908 089). Primer combinations were selected for further use where they produced a large number of polymorphic, reproducible and easy-to-score markers.

The selective AFLPs were then performed with the 4 optimised primer combinations; all primers had a three base pair extension (EcoR1 5'-GAC TGC GTA CCA ATT CAA C+3, +ATC or +AAC; Mse1 5'-GAT GAG TCC TGA GTA ACT+3, +CTA or +CTT). The reaction mix was as follows: 1µl diluted pre-amp product, 0.8µl 10x PCR buffer, 0.64µl $MgCl_2$ (25mM), 0.8µl dNTP's (2mM), 10µl Eco R1 primer (10µM) , 10 µl Mse1 primer (10µM), 3.68µl dH20, 0.08µl Hot Start™ Taq Polymerase (2.5 unit/µl), 0.5µl BSA (0.045mg/ml). The primers were from MWG Biotech AG™; PCR reagents were from Sigma™, except BSA and dNTP's which were from Bioline™. The PCR program was as follows: initial denaturation at 94ºC for 2 minutes, followed by 30 cycles in total of: 94ºC for 20 seconds, with 10 amplification cycles at 66-56ºC touchdown, then 20 cycles at 56ºC with a primer extension time of 72ºC for 1 minute, then a final extension time at 60ºC for 30 minutes.

One 1µl of product with 0.66µl size standard (Beckman Coulter™) and 35µl of sample loading solution (Beckman Coulter™) per sample was loaded on a Beckman Coulter CEQ8000 automated sequencer.

2.2.4 AFLP data collection and scoring

2.2.4.1 Data collection

The CEQ8800 software (Beckman Coulter Inc.) was used to automatically create a data matrix, which was then edited manually. Fragments that could not be scored unambiguously in all samples were deleted from the analysis. No fragments under 1000 fluorescence units' intensity were scored. In the initial data editing, fragments under 100bp were deleted for two reasons, firstly because they were difficult to score due to large numbers of fragments in overlapping size classes and secondly, because homoplasy is greater in smaller sized fragments (Vekemans *et al*. 2002). This cut off point was arbitrary, as was the cut off point of 1000 fluorescence units for intensity.

2.2.4.2 Testing AFLP scoring consistency

In order to measure scoring consistency, the data from one primer pair was scored twice and the results of the scoring were compared. The percentage error was calculated as the number of fragment presences scored differently between the two datasets multiplied by 100.

2.2.4.3 Test duplicated fragments for different primers pairs

The choice of primer pairs that produced good traces was very limited. The primer pairs selected after screening were as follows: EcoAAC.MseCTT; EcoAAC.MseCTA; EcoAGC.MseCTT; EcoAGC.MseCTA. One potential problem was that the primer pairs all had reciprocal duplications of primers between them, so there was a chance that the same Eco fragments could appear in the output for more than one primer pair. In order to test for the presence of duplicated fragment, ten samples were chosen and the traces were overlaid and compared to check for duplications.

2.2.5 Statistical analysis of AFLP data

2.2.5.1 Analysis of AFLP fragment frequency.

The data generated from the four primer pairs was pooled together to form one dataset. The numbers of fragments in the following groupings were calculated: individuals, populations, regions, taxa, ploidy level. The putative hybrid populations from Greenfield were excluded from this analysis. The variation in the number of markers between pre-defined groups was also tested using a nested General Linear Model Analysis of Variance (GLM ANOVA). The software package Excel™ was used to organise the dataset and count the number of fragments within groups. ANOVA analysis was done using the software package MINITAB® 14 (Minitab Inc).

2.2.5.2 Analysis of genetic variation, based on AFLP fragments.

The sampling of two individuals per population allowed the variation in many populations to be compared in one dataset. However, the small number of individuals per population meant that gene diversity could not be accurately calculated. The number of polymorphic loci per population was calculated as a measure of diversity using the software package Arlequin 3.1 (Schneider *et al*. 2000).

The data were divided into two categories, pairs of individuals in the same population, and all other pairings between individuals in different populations. Jaccards pairwise similarity was calculated for each of these pairs using the software package Multivariate Statistical Package 3.13 (MSVP: Kovach 1999). The difference between the similarities of pairs in the same populations and pairs in different populations was tested using a T-test in MINITAB™.

To assess patterns of variation and clustering without pre-defining groups, principal co-ordinate (PCO) analysis was undertaken using MVSP. The PCO analysis was based on Jaccard's similarity co-efficient converted to Eigenvectors and presented as a scatter plot. In

order to see how the variation was distributed between pre-chosen groups, an AMOVA analysis was undertaken using Arlequin 3.1. All AMOVA analyses estimated variance among populations. In different analyses, populations were nested either between taxa, regions (N England, SW Scotland, NW Scotland, E Scotland, Wales), chromosome numbers or habitats (mountain or coast) in separate runs.

2.3 Results

2.3.1 Quality control outcomes and results

2.3.1.1 Percentage error for primer pair dataset scored twice

There were 15 fragments that were different in presence or absence between the two scored datasets, which comprised 491 fragments in total. The percentage error caused by scoring was calculated as (15/491)*100 = 3.05%.

2.3.1.2 Checking for duplicated Eco fragments

Of the 20 pairs checked, there were no duplicated Eco fragments found in the traces. Therefore, if they exist in the dataset, they were assumed to be at a very low frequency.

2.3.1.3 Duplicated sample to test for within-plate reproducibility

There were some small differences in peak shape between the duplicated samples (shown in Figure 2.2), but the traces yield the same fragments. The traces showed poorer reproducibility for fragments of less than 100bp. These fragments were automatically eliminated from the dataset and so did not influence the results

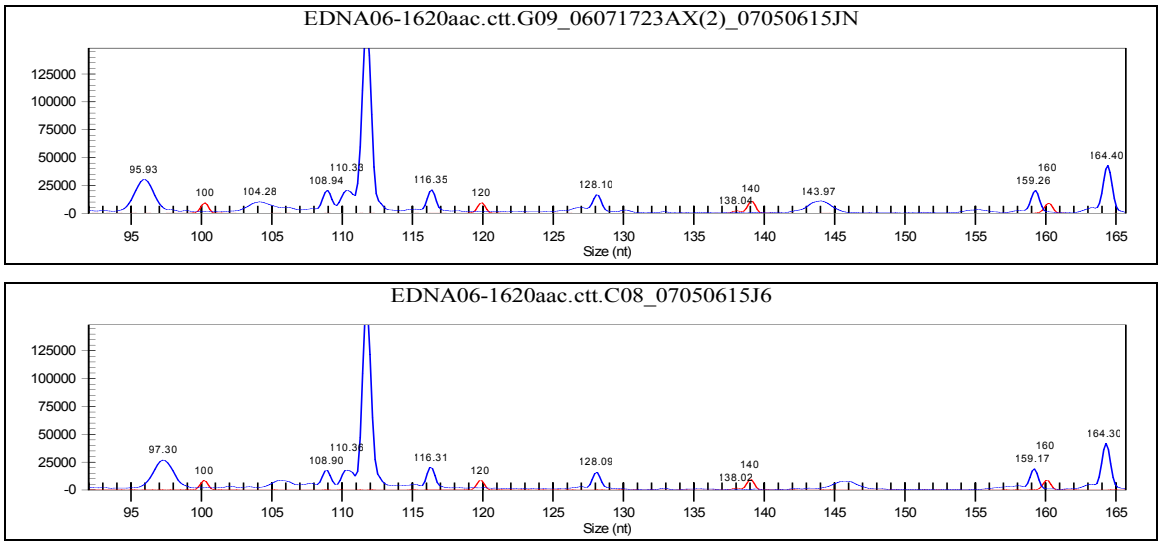

Figure 2.2: Shows the AFLP electropherograms of the duplicated sample MG8 in the dataset. The more similar the electropherograms are, the more reproducible the results are judged to be.

2.3.2 Fragment frequency results

AFLP analysis on the 68 accessions in this dataset produced 262 scorable fragments. The number of AFLP fragments produced varied between 56.0 and 62.0 among different populations (Table 2.2). The number of AFLP fragments (Table 2.2) derived from samples of coastal populations (61.7) was lower than that for samples of upland populations (57.3). The number of AFLP fragments produced decreased with increased ploidy level, from 64.0 to 54.4 (Table 2.2). Private and diagnostic markers analysis between populations is not shown because only two samples per population were used. One population, Cawbank Spring, had a fragment that was not present in other populations. There were no diagnostic fragments found for the groupings taxa, habitats, region or ploidy level.

2.3.2.1 ANOVA for fragment number between pre-defined groups

Fragment number varied significantly among populations in all the analyses (Tables 2.3-2.5). The number of fragments did not vary significantly between habitats (Table 2.3), regions (Table 2.4) or ploidy level (Table 2.5).

Region	**Mean average fragment number**
N. England	61.7
Mid-N. Wales	61.4
E. Scotland	59.7
N. W. Scotland	62.0
S. W. Scotland	59.9
S. Wales & England	57.0
Mid Scotland	56.6
Habitat	
Upland	57.3
Coast	61.7
Ploidy level	
12	64.0
24	60.0
26	58.1
42	54.4

Table 2.2: Mean average AFLP fragment number produced from *Cochlearia* samples of seven taxa from different regions, habitats and ploidy levels.

Source	DF	MS
UplandvCoast (habitat)	1	334.12
Between populations within habitats	32	161.22**
Error	34	63.54
Total	67	

Table 2.3: Showing results of GLM nested ANOVA testing for significant differences in the number of AFLP fragments produced between *Cochlearia* populations nested within upland or coastal groups. (* = P value < 0.05, ** = P value < 0.01, *** = P value 0.001)

Source	DF	MS
Region	6	44.11
Between populations within regions	27	193.65**
Error	34	63.54
Total	67	

Table 2.4: Showing results of GLM nested ANOVA testing for significant differences in the number of AFLP fragments produced between *Cochlearia* populations nested within region. (* = P value < 0.05, ** = P value < 0.01, *** = P value 0.001)

Source	DF	Adj MS
Chromosome number	3	54.74
Between populations within chromosome number	29	182.82**
Error	33	61.59
Total	65	

Table 2.5: Showing results of GLM nested ANOVA testing for significant differences in the number of AFLP fragments produced between *Cochlearia* populations nested within chromosome number.

2.3.3 Genetic data analysis

In the PCO plot (Figure 2.3), only a small amount of the total variation was accounted for by axis 1 & 2 (6.24% and 5.61% respectively). The PCO plot shows that individuals within the same population tended to be more similar to each other than any random pair of individuals. This was supported by the analysis of the pairwise similarity results: the mean average similarity between individuals in the same population was 0.51 (Jaccard's similarity co-efficient) and the mean average for individuals in different populations was 0.35 (Jaccard's similarity co-efficient). The T-test was used to test the difference between the average similarity within populations and the average similarity between any other pair in the dataset. The results for this calculation were: t = -8.24, p < Value = 0.001, DF = 33. The paired

samples from within populations were highly significantly more similar than pairings between populations.

All the AMOVA analyses showed variation among populations (Tables 2.6-2.9). There was no significant variance in genetic data between groupings with different ploidy levels (2.6), among groupings from different regions (Table 2.7), between groupings from the different habitats (Table 2.8) or among different taxa (Table 2.9).

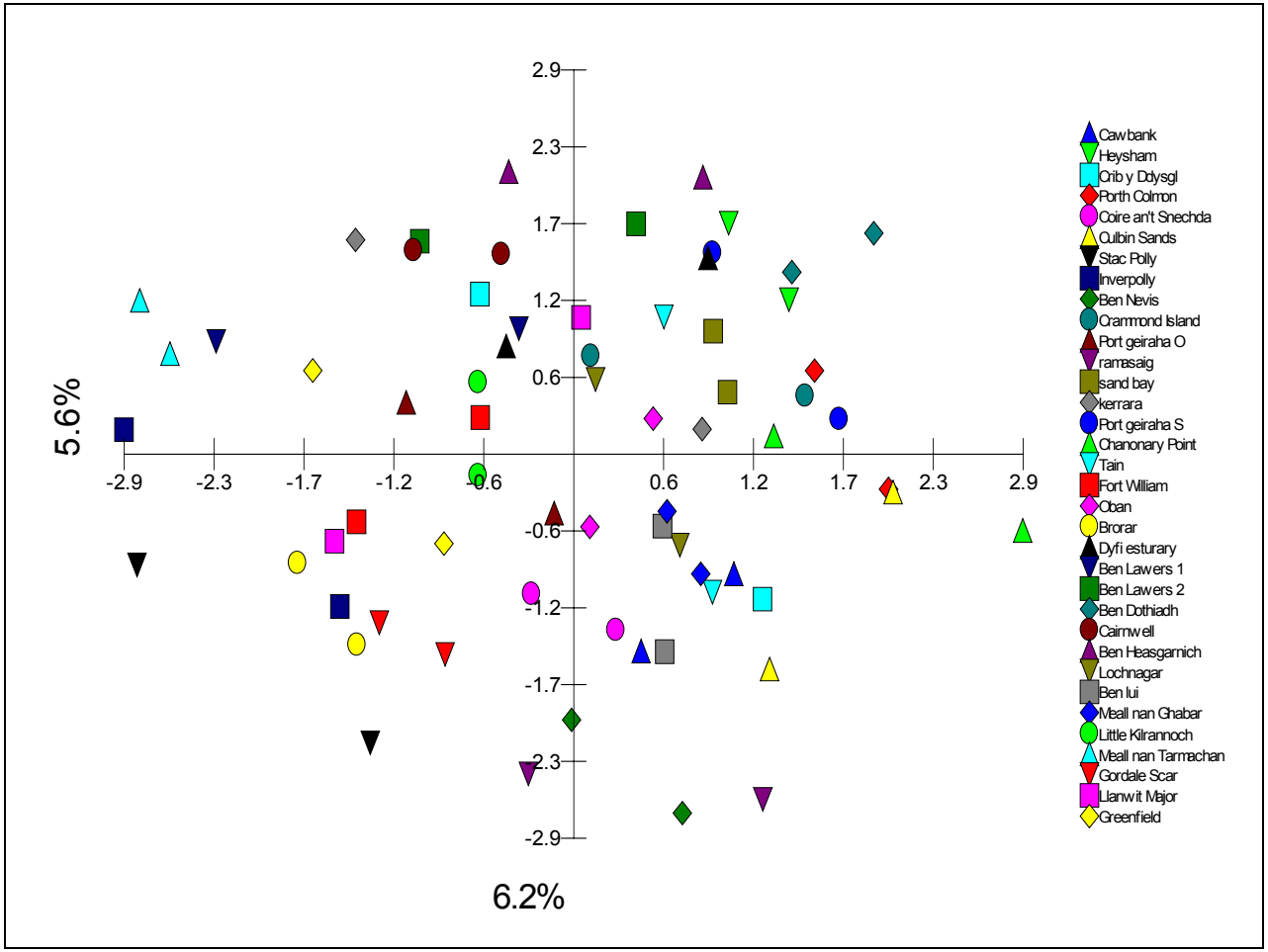

Figure 2.3: A PCO plot showing the phenetic relationships between all sampled individuals from 34 *Cochlearia* populations of different taxa (populations shown in different colours) based on AFLP data converted to Jaccard's similarity.

Source of variation	D.F	Sum of squares	Variance	% of variation	P-values
Among ploidy levels	3	1.51	-0.0015	-0.31	0.26
Among pops. within ploidy levels	29	14.98	0.0158	3.17	<0.0001
Within populations	33	16.00	0.4849	97.13	

Table 2.6: AMOVA of AFLP data to test for significant genetic variation between populations with different chromosome numbers, among populations with the same chromosome number.

Source of variation	D.F	Sum of Squares	Variance	% of variation	P-values
Among regions	6	3.11	0.0004	0.10	0.14
Among pops. within regions	27	13.88	0.0143	2.86	<0.0001
Within populations	34	16.50	0.4820	97.05	

Table 2.7: AMOVA of AFLP data to test for significant genetic variation between populations of different regions, among populations within regions.

Source of variation	D.F	Sum of squares	Variance	% of variation	P-values
Among coastvupland (habitats)	1	0.51	-0.0001	-0.01	0.47
Among pops. within habitats	32	16.47	0.0147	2.95	<0.0001
Within populations	34	16.50	0.4853	97.06	

Table 2.8: AMOVA of AFLP data to test for significant genetic variation between populations from different habitats and among populations of different habitats.

Source of variation	D.F	Sum of squares	Variance	% of variation	P-values
Among taxa	5	2.54	0.0009	-0.19	0.70
Among pops. within taxa	27	13.95	0.0159	3.18	<0.0001
Within populations	33	16.00	0.4849	97.01	

Table 2.9: AMOVA of AFLP data to test for significant genetic variation between taxa and among populations within taxa.

2.4 Discussion

2.4.1 AFLP quality control

The AFLP method produced good quality traces. There did not seem to be major problems with fragment reproducibility, reproducibility in scoring or with duplicated Eco-Eco fragments. It is highly unlikely that the level of error in these variables would significantly change the relationships or the'biological answers' inferred from the results. The level of homoplasy within the data set was not known, but was assumed to be low as low molecular weight fragments were excluded.

2.4.2 Overall patterns of genetic variation

Overall, the dataset is very 'noisy', with very little structure beyond that seen among populations. It is likely that a clearer picture will emerge with more intensive sampling of populations targeted at answering specific questions. The lack of diagnostic fragments reflects the overall lack of structure and differentiation in the data.

There were no significant differences in fragment number (Table 2.5) between populations with different ploidy levels. This may be because the tetraploids are autopolyploids rather than allopolyploids. If the tetraploid 2n = 24 originated by autoploidy from the diploid 2n = 12, then the effect on the AFLP traces may simply be to increase peak height of the fragments, because of the extra copies of each fragment.

There was no significant genetic differentiation between populations with different chromosome numbers (Table 2.6). This is in stark contrast to the results from a study of the complex polyploid group *Euphrasia* in Britain, where 25.1% of the AFLP variation was partitioned between diploid and tetraploid populations *Euphrasia* (French 2003). The lack of differentiation in AFLP fragments between plants of different chromosome numbers in *Cochlearia* suggests that there was not a strong barrier to gene flow between plants of different chromosome numbers; this was backed up by previous crossing experiments in *Cochlearia* (Table 1.2 - Saunte 1955, Gill 1973, Nordal & Laane 1990, Koch *et al.* 1996). Gene flow between different ploidy levels has also been recorded in *Draba*, also a member of the family Brassicaceae (Brochmann *et al.* 1992).

The lack of regional structuring was surprising because very clear geographical structuring was found using AFLPs for studies of *Cochlearia* populations in the Carpathian Mountains

(Koch 2002, Kochjarová *et al.* 2006). This may be because the Carpathian studies were on a much larger geographical scale than this study. A lack of geographical structure in genetic data is not unique, especially among populations of post-glacial re-colonisers from single or closely related source populations (Jørgensen & Mauricio 2004). A similar lack of geographical structuring was found in *Silene tatarica* (Tero *et al*. 2003), *Trollius europeaus* (Després *et al*. 2002), *Euphrasia stricta* (Kolseth & Lönn 2005) and *Eryngium alpinum* (Gaudeul *et al.* 2000). Similarly, a study of *Arabidopsis thaliana*, introduced to North America by humans multiple times also shows lack of geographical structure.

2.5 Conclusions

There were significant differences in genetic variation between populations, but no significant differences among ploidy levels, regions, habitats or taxa (Tables 2.6-2.9). The differences in ploidy level, which have strongly influenced *Cochlearia* taxonomy in recent years, do not appear to form a barrier to gene flow. There was no evidence of genetically separated lineages. This does not rule out the possibility of divergence in adaptive traits that cannot be detected by AFLP analysis. These initial results indicate that the *Cochlearia* in Britain have diversified recently from the same source.

The chapters that follow will focus on specific populations and questions. Using larger sample sizes and combining AFLP data with other techniques, more information will be gathered about the way in which variation is partitioned among the British *Cochlearia.*

3. Morphological and genetic diversity in coastal *Cochlearia*

Abstract

Two British endemic taxa with a coastal distribution have been described within the *Cochlearia officinalis* s.l. complex in Britain. They are often hard to distinguish from each other and from other members of the complex. This chapter aims to investigate whether there are discrete genetic or morphological groups within the assemblage of coastal *Cochlearia officinalis* s.l. Three species of the *Cochlearia officinalis* s.l. complex were sampled: the two putative endemics *C. atlantica* and *C. officinalis* subsp. *scotica* and the widespread *C. officinalis* s.s. Each taxon was sampled from four populations around the coast of Britain. The samples were screened for correlated sets of morphological characters and AFLP markers to test whether they corresponded with taxonomic groupings. In some cases variation in individual morphological characters correlated with taxonomic groupings. However, when the morphological characters were combined they did not distinguish groups. Populations of the same putative taxonomic group from different populations did not group together according to genetic similarity.

3.1 Introduction

There are two putative rare endemic taxa within the assemblage of Scottish coastal *Cochlearia officinalis s.l.* These are *C. officinalis* subsp. *scotica* (Druce) P.S. Wyse Jackson (=*C. scotica* Druce) and *C. atlantica* Pobed. These two taxa can be difficult to distinguish from each other and they both intergrade with *Cochlearia officinalis* s.s., a species that is widespread across Europe. *Cochlearia officinalis* subsp. *scotica* (or *C. scotica*) has a species action plan, but its status is 'taxonomically uncertain' (UK Biodiversity Steering Group 1995). *Cochlearia atlantica* is a potential endemic species, classified under ICUN criteria as 'data deficient'. The conservation policy for *Cochlearia* cannot move forward until the taxonomy is resolved, and the aim of this chapter was to obtain the data needed to clarify the taxonomy of coastal *Cochlearia* and to develop appropriate conservation recommendations.

3.1.1 Ecological diversity among coastal *Cochlearia*.

The *Cochlearia officinalis* s.l. complex occupies a range of niches in the coastal ecosystem: shingle and sand beaches, sand dunes, coastal grassland, saltmarsh, brackish marsh and bird

cliffs. One of the greatest differences between these niches is nutrient level. Nordal & Stabbetorp (1990) recorded very low nutrient levels for the shingle habitats, but very high levels of nutrients in the bird cliff habitats. Differences in *Cochlearia* populations growing in different habitats have been noted. *Cochlearia officinalis* s.s. growing on bird cliffs have an increased ability to metabolize nitrogen using nitrogen reductase compared with other populations (Odasz 1994, Nordal *et al.* 1986). The seeds of coastal *Cochlearia* that grow below the high tide mark germinate in the autumn, as soon as they are shed, and this is thought to allow the seedlings to become established before high spring tides wash the seeds away (Pegtel 1999). In general, coastal cytotypes can tolerate and germinate in higher NaCl concentrations than inland ecotypes (Pegtel 1999)

3.1.2 Mechanisms of divergence and adaptation

The ability of *Cochlearia* to live in all these habitats requires genetic adaptations or phenotypic plasticity in response to the environment (Hurka & Neuffer 1997). Phenotypic plasticity means that individuals can respond physiologically to all the habitats in which they grow without genomic change under selection. In reality, the absence of genetic change caused by differential selective pressures unlikely considering the varied habitats in which *Cochlearia* grow. Local genetic adaptation can occur quickly and does not necessarily require genetic isolation of populations, individuals or of the whole genome of an individual (Morjan & Rieseberg 2004). Adaptive genetic change can be restricted to a few loci or involve additive changes in allele frequency across many loci (Morjan & Rieseberg 2004). Adaptive changes in the frequencies of existing alleles can occur with each generation under selection. New mutations appear stochastically and take longer to accumulate, although if they are favourable to survival, they will spread quickly.

Although local adaptation is a highly influential speciation process, it can be difficult to detect experimentally. Some studies have used AFLP to search for loci with extreme Fst values, inferring that these are under selection pressures (Campbell & Bernatchez 2004). This approach must be applied with caution as extreme values are expected by chance, and are not necessarily a result of selection. The behaviour of loci in response to selection cannot be clearly established without genetic mapping or gene expression studies. The phenotypic expression of genetic adaptation to different habitats can be detected using transplant experiments (Bauert 1996, Kik *et al.* 1990, Stanton & Galen 1997) or by growing plants under standard conditions (Bauert 1996, Nordal & Stabbetorp 1990). In *Cochlearia* neutral markers can be used to test whether genetic similarities are greatest between populations

with similar morphologies and/or ecologies, or whether genetic similarity is explained by geographical proximity. If putative species are 'good species' they should form genetic groupings, irrespective of their geographic origin. However, if they are just locally adapted ecotypes, they should show greater genetic affinities to morphologically dissimilar, but geographically proximal populations.

3.1.3 Gene flow and isolation by distance

If *Cochlearia* populations are comprised of only one taxon, then we would expect gene exchange to occur between them. If populations exchange genes freely, a pattern of isolation by distance will develop over time. In an isolation-by-distance model, gene flow is greatest between nearby populations, with populations at the extremes of the range being least similar. In some cases, isolation-by-distance will not be seen even if populations are of the same taxon and theoretically able to exchange genes. For example, if local gene flow is no greater than gene flow between distant populations (Gaudeul *et al*. 2000). Gene flow between nearby populations may be prevented by landscape barriers or lack of pollinators. An isolation by distance pattern may not be seen if colonisation has been recent and unstructured (Després *et al*. 2002). A drift – migration equilibrium will only become established after sufficient generations of gene flow between populations have occurred.

3.1.4 Existing work on the taxonomy of coastal *Cochlearia*: morphological characters

Traditional taxonomic work, in the field and herbarium has identified characters to distinguish the three coastal taxa in Scotland (Table 3.1). *Cochlearia atlantica* can be distinguished primarily by its truncate-based leaves and rosette habit. *Cochlearia officinalis* subsp. *scotica* is generally a very small cushion forming plant with angular petals. *C. officinalis* s.s. is a much larger, more robust plant with cordate leaves.

Taxon	Height	Leaves	Petal size (mm)	Petal colour	Petal shape	Habitat
***C. officinalis* s.s.**	5-50cm	Ovate-reniform	3-7	White	Narrow, short claw	Flushed grass, upper saltmarsh, cliffs
C. officinalis* subsp. *scotica	1-5cm	Cordate-truncate	3-4	White or pinkish	Squared blade, long claw	Grassland, rocks, shingle
C. atlantica	5-20cm	Ovate-cordate, often purplish	2-4	White	Short claw	Lower saltmarsh, shingle

Table 3.1 Distinguishing morphological characters for the three Scottish coastal taxa, amalgamated from taxonomic accounts (Dalby 1996, Stace 1997).

Although *Cochlearia* taxa might appear relatively easy to distinguish based on their descriptions, ambiguous populations and individuals are often encountered. Studies on *Cochlearia* have highlighted the difficulties in delineating taxa among the assemblage of coastal *Cochlearia* (Hultén 1970, Nordal & Laane 1996). Morphological characters do not allow the clear delineation of species and many defining characters could be partly influenced by environmental differences. The influence of environmental variables (particularly light intensity) on the size and shape of basal leaves and inflorescence branching were noted by Nordal & Stabbetorp (1990). *Cochlearia* adapted to low nutrient levels often grow slowly, independent of the nutrient levels, to conserve resources (Nordal *et al.* 1986). This adaptation could result in small and large phenotypes of the same species that are maintained in cultivation.

In order to investigate this, Nordal & Laane (1990), Nordal & Stabbetorp (1990) and Pegtel (1999) grew Scandinavian *Cochlearia* from seed in phyto-chambers. A morphometric study of these plants showed that certain morphologies were associated with certain ecological situations. They found that differences in petal length, leaf-base angle, seed size and growth form were maintained between ecotypes and ploidy levels under standardised conditions. Morphometrics have proved useful in clarifying the taxonomy of the complex Brassicaceae group *Cardamine pratensis* (Marhold *et al.* 1993, Lihova *et al.* 2003). Morphometric techniques have never been applied to British *Cochlearia*. Morphometric techniques can be especially useful for taxonomic clarification combined with other types of data (e.g. molecular).

3.1.5 Existing work on the taxonomy of coastal *Cochlearia*: cytological work

All the chromosome counts taken from UK coastal populations within the *C. officinalis* complex have been 2n = 24 (tetraploid), (Gill 1971), so the differences between coastal taxa are not the result of different ploidy levels. Although occasional hybridisation with *C. danica* (2n = 42) or *C. anglica* (2n = 36) producing chromosomal variants cannot be ruled out (Stace 1975).

3.1.6 Question

Can populations defined as *C. officinalis* subsp. *scotica*, *C. atlantica* and *C. officinalis* s.s. be distinguished from each other using a) morphological characters b) AFLP markers c) both combined?

3.1.7 Approach

A combination of molecular and morphological techniques is a powerful way to define taxonomic groups (Hillis 1987, Fjellheim 2001). Four populations were chosen from each of the three taxa, *C. officinalis* subsp. *officinalis*, *C. officinalis* subsp. *scotica*, *C. atlantica*. In the case of the two endemic species, samples were taken from the two type locations, then other populations were selected that were most similar in morphology and ecology to the described species and the plants at the type location. The AFLP technique was used to provide markers from across the genome. A suite of morphological characters were chosen from those that had proved useful in previous studies and that could be measured throughout the field season in all populations. Then the three sets of populations were screened for AFLP marker variation and morphological character variation. If three distinct taxa are present then congruent groupings of genetic similarity and morphology are expected. Thus if the variation in AFLP markers and morphological markers reveal the same groupings, it would provide good support for the existence of separate species.

3.2 Methods

3.2.1 Sampling methods

Four populations each of the three putative taxa were chosen from a range of habitats and geographical locations for morphological and genetic analysis (Table 3.2). The distribution of these populations is shown in Figure 3.1. The sampled populations were grouped into four different geographical regions. These regions were East Scotland, Wales & England, South West Scotland and North West Scotland. Plants from the type locations of *C. officinalis* subsp. *scotica* and *C. atlantica* were included. The identity of individuals from all populations was verified by Tim Rich, either in the field or using herbarium specimens. A list of associated species was recorded for each population.

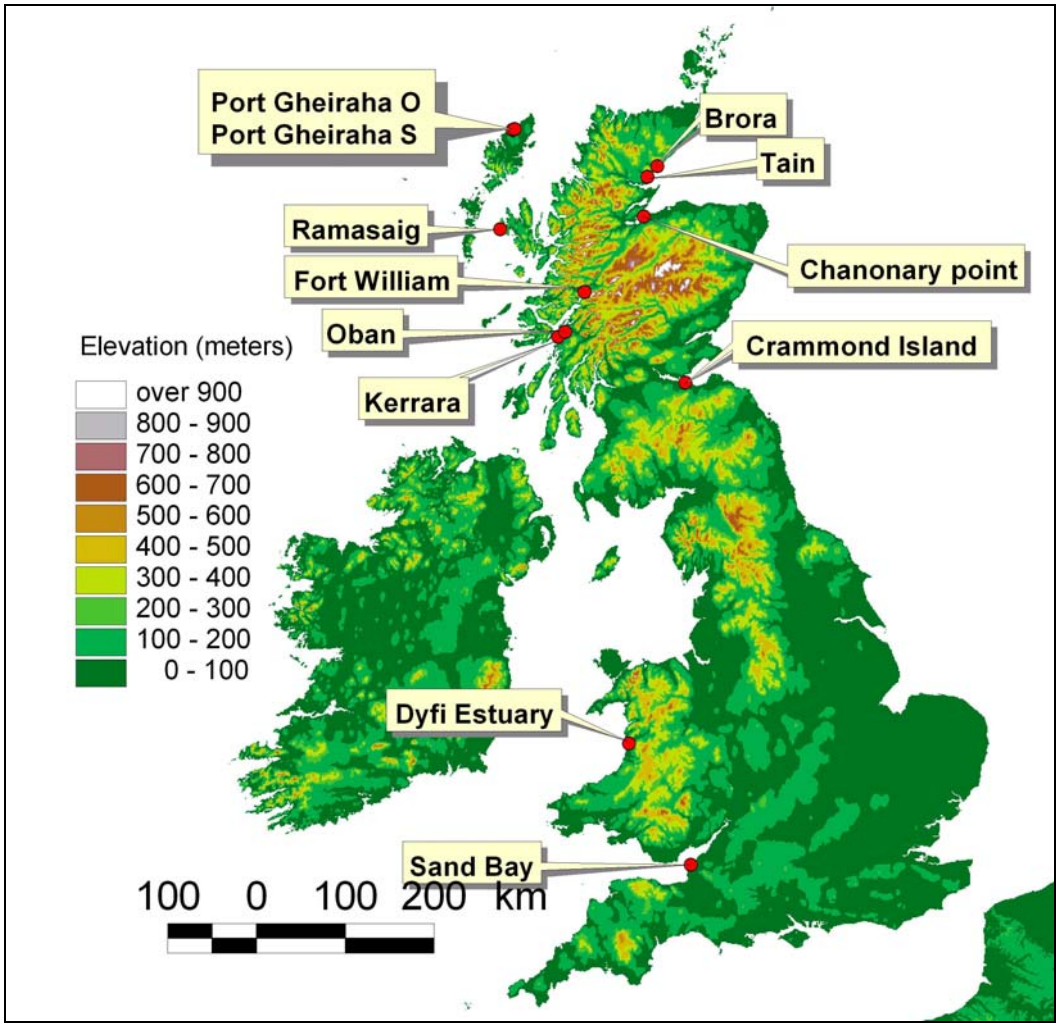

Figure 3.1: A map of Britain showing the locations of twelve populations of three taxa coastal taxa in the species complex *Cochlearia officinalis* s.l.

Region and NT grid ref.	Site name	Habitat and associated species	Number of samples	
			AFLP	Morph
***C. officinalis* s.s.**				
E Scotland NT/200.784	Crammond Island	**Grassland, shingle**, *Trifolium repens, Galium aparine, Tripleurospermum inodorum* subsp. *maritimum, Plantago lanceolata, Dactylis glomerata, Festuca ovina, Armeria maritima*	**8**	**12**
Wales/ England ST/322.630	Sand Bay,	**Limestone cliffs**, *Ulex europeaus, Plantago lanceolata, Hedera helix, Rubus fruticosus, Festuca rubra, Geranium robertianum*	**7**	**10**
NW Scotland NG/159.436	Ramasaig,	**Shingle, upper shore**, *Armeria maritima, Festuca rubra* nearby	**8**	**15**
NW Scotland NB/336.497	Port Gheiraha O	**Bird cliff**, *Festuca rubra, Taraxacum officinalis, Armeria maritima, Sedum rosea, Ligusticum scoticum, Ranunculus acris*, *Agrostis stolonifera*, *Tripleurospermum inodorum subsp. maritima*	**7**	**15**
C. atlantica				
Wales/ England SN/617.934	Dyfi Esturary,	**Esturary saltmarsh**, *Elytrigia repens, Festuca rubra, Aster tripolium, Glaux maritima, Plantago maritima, Juncus gerardi, Rumex crispus*	**8**	**7**
SW Scotland NM/862.329	North Oban	**Rocks, upper shore**, *Plantago coronopus, Sedum anglicum, Lychnis flos-cuculi, Potentilla anserina, Lotus corniculatus, Plantago lanceolata, Plantago maritima, Festuca ovina, Armeria maritima*	**8**	**10**
E Scotland NC/908.089	Brora	**Saltmarsh, mouth of river**, *Plantago lanceolata, Festuca ovina, Lolium perenne, Armeria maritima*	**8**	**15**
SW Scotland NN/087.764	Loch Linnhe, (Fort William)	**Shingle and sea wall**, *Taraxacum officinale, Trifolium repens, Plantago coronopus, Spergularia media*	**8**	**8**
C. officinalis* subsp. *scotica				
E Scotland NH/747.556	Chanonary Point	**Shingle, upper shore**, *Hieracium agg. Suaeda maritima, Trifolium repens, Holcus lanatus, Galium aparine, Tripleurosperum inodorum subsp. maritimum, Puccinellia maritima, Festuca ovina, Lolium perenne*	**8**	**15**
SW Scotland NM/802.264	Kerrara	**Grazed headland**, *Festuca rubra, Cerastium fontanum, Koeleria macrantha, Sagina nodosa, Thymus polytrichus, Cynosurus cristatus, Silene vulgaris subsp. maritima, Plantago coronopus,*	**8**	**10**

		Plantago lanceolata, Plantago maritima, Festuca ovina, Armeria maritima		
E Scotland NH/794.973	Tain	**Saltmarsh, mouth of river**, *Plantago maritima, Suaeda maritima, Festuca ovina, Glaux maritima, Armeria maritima, Puccinellia maritima, Spergularia media, Aster tripolium*	**8**	**14**
NW Scotland NB/335.499	Port Gheiraha S	**Rocks and grazed grassland**, *Festuca ovina, Armeria maritima, Rumex acetosella, Plantago martima*	**8**	**10**

Table 3.2: Sampled population locations of three coastal taxa in the complex *C. officinalis* s.l. including associated plant species and number of samples taken for morphological and AFLP marker analysis.

3.2.2 Methods for plants grown in greenhouses

Seeds were collected when available from the sampled populations (the plant from Tenby was collected as an adult). These seeds were sown in potting compost and grown in glass house under standard conditions. There were not enough individuals to make statistical comparisons between plants from different populations. Nevertheless, they formed a useful collection from which informal comparisons of morphology could be made between plants growing in a common environment.

3.2.3 Morphological measurements

The largest rosette leaf and the largest petal were taken from 10-20 plants in each of the sampled populations (Table 3.2). A voucher specimen for every population was deposited in Royal Botanic Garden Edinburgh Herbarium (E). The leaves were pressed and the petals preserved under tape for measurement. Five morphological characters were measured and recorded: leaf length, leaf width, leaf base angle (see Figure 3.2), petal length, petal width; the ratios between the length and width of petals and leaves were also calculated. The morphological characters that could be used were limited to those that could be measured on samples collected throughout the field season. It was not possible to measure seed pod characters as seed pods are only present for a short time towards the end of the field season.

3.2.4 Port Gheiraha

Two morphologically distinct sub-populations occupying distinct habitats (cliff *vs* coastal turf) were encountered at one site (Port Gheiraha). The plants on the cliff had the morphology of *C. officinalis;* those on the coastal turf had the morphology of *C. officinalis* subsp. *scotica*. To test whether plants of different morphologies were differentiated from

each other when growing in close proximity, these sub-populations were included in the main analyses, but also analysed separately.

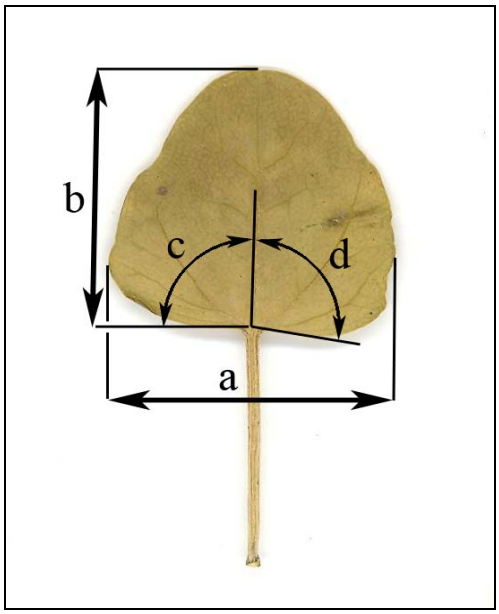

Figure 3.2: A diagram showing how the leaf measurements were made, a: width, b: length, c: leaf base angle 1, d: leaf base angle 2. The mean of the two leaf base angles was used in the analysis.

3.2.5 AFLP marker generation

The coastal populations were scored for AFLP marker loci based on the samples listed in Table 3.2. The methods for DNA extraction and AFLP marker generation were as described in the chapter 2.

3.2.6 Data analysis: morphology

Boxplots were created in the software package MINITAB® 14 (Minitab Inc.) to show the variation within and between populations and taxa for each morphological character. Nested general linear model (GLM) analysis of variance (ANOVA) was used to calculate the variance in morphological characters among regions and among populations; among taxa and among populations. The percentage of the variation attributed to each grouping was then calculated from the variance components. In order to see whether the combined morphological characters separated the taxa, the morphological measurements data was converted to principal component scores using the Multi-Variate Statistical Package 3.13

(MVSP: Kovach, 1999), then the axis one and two PCA scores were plotted against each other in MINITAB®. The scores on the first PCA axis were also analysed using a GLM ANOVA to see how variation was partitioned between different groupings.

3.2.7 Data analysis: AFLPs

The data from the four primer combinations was pooled together to form one dataset. Fragments present in only one group (private fragments) and fragments present in all the individuals within a group, but in no other groups (diagnostic fragments) were counted. The number of private fragments and shared fragments between the two populations at Port Gheiraha were also recorded. The variation in the number of fragments produced between groups was analysed using GLM nested ANOVAs in MINITAB®. The groupings for these ANOVAs were as follows: (1) between regions and among populations within regions; (2) between taxa and among populations within taxa. The number of fragments polymorphic at the 5% level was calculated using AFLP-SURV (Vekemans et al 2002).

Principal co-ordinate analysis (PCO) was used to assess patterns of variation and clustering within the data. PCO scores were generated from Jaccard's Pairwise Similarity Coefficient in MVSP. The first two axes in the data were then plotted as a scatterplot. In order to see how the variation was distributed between pre-chosen groups an AMOVA approach was used in Arlequin 3.1 (Schneider *et al*., 2000). The groupings were: (1) between regions and among populations within regions; (2) between taxa and among populations within taxa. Pairwise Φst's (based on AMOVA) between populations and average values across populations were calculated using Arlequin. A Mantel test (Mantel 1967) was performed using Arlequin to detect whether there was a correlation between geographical distance and genetic distance in the data.

3.3 Results

3.3.1 Results from morphological studies

3.3.1.1 Plants grown from seed

The *C. officinalis* subsp. *scotica* plants collected from coastal grassland on the Scottish Islands of Helliday and Fiaray (left Figure 3.3, top-right Figure 3.5) maintained their compact growth form and small, rounded cordate leaves when grown in the glasshouse. This taxon from these sites also consistently produced short inflorescences. Their seedlings also maintained the same compact growth form (although only eighteen month's growth was observed). The *C. officinalis* subsp. *scotica* collected from shingle at Inverpolly, North-West Scotland (bottom left Figure 3.5) had a rosette growth form, rather than a cushion shaped growth form as seen in the Fiaray and Hellisay plants. The plant size and leaf shape however, conformed to the taxonomic description given for *C. officinalis* subsp. *scotica.* The plants from shingle at Little Ferry, East Scotland (bottom right Figure 3.5) were identified as *C. officinalis* subsp. *scotica* in the field. When they were cultivated in the glasshouse, they grew much larger and took on the appearance of *C. officinalis* s.s. The taxonomic group to which this population belongs was ambiguous.

C. officinalis s.s. collected from coastal cliffs at Tenby, Wales (right Figure 3.3) grew more vigorously in the glasshouse, but the leaves, flowers and seed pods did not change in size. The flowers were produced on long trailing inflorescences. The plants from saltmarsh at Findhorn bay, Eastern Scotland had typical *C. atlantica* (left Figure 3.4) morphology, with a rosette shape and a leaf base angle of around 90% (therefore truncate). The leaves also have purple veins and blotches, a character often seen in more northerly *Cochlearia* populations in Britain. The characteristics of the plants in the wild population were maintained in the glasshouse. The plants collected at Rhiconich, N.W. Scotland (top left Figure 3.5) were intermediate between *C. atlantica* and *C. officinalis* subsp *scotica*.

Figure 3.3: *C. officinalis* subsp. *scotica* from Fiaray, W. Scotland (left); *C. officinalis* from Tenby, Wales (right).

Figure 3.4: A plant with morphology intermediate between *C. officinalis* subsp. *scotica* & *C. atlantica* from Rhiconich, W. Scotland (left); *C. atlantica* from Findhorn Bay, E. Scotland (right).

Figure 3.5: A plant with morphology intermediate between *C. officinalis* subsp. *scotica* and *C. atlantica* from Rhiconich, N.W. Scotland (top left); *C. officinalis subsp. scotica* from Hellisay, W. Scotland (top right). *C. officinalis* subsp. *scotica** from Little Ferry, Eastern Scotland (bottom right); *C. officinalis* subsp. *scotica*, Inverpolly, N.W. Scotland. * This was named *C. officinalis* subsp. *scotica* in the field, because it was very small in the field and growing on shingle; however in cultivation it appears to be a specimen of *C. officinalis* s.s.

3.3.1.2 Morphological analysis of leaves and petals collected from wild populations

The box-plots (Figures 3.6-3.12) showed a great deal of overlap in variation between taxa for leaf length (Figure 3.6), leaf width (Figure 3.7), leaf length:width ratio (Figure 3.8), leaf base angle (Figure 3.9), petal length (Figure 3.10), petal width (Figure (3.11) and petal length-width ratio (Figure 3.12). The variation among populations of the same taxon was greater than the variation between taxa. *C. officinalis* subsp. *scotica* tended to have shorter, narrower leaves (Figures 3.6 and 3.8) and petals (Figures 3.10 and 3.11) than *C. atlantica* or *C. officinalis* s.s. There was little difference in the leaf length: width ratio (Figure 3.7) or petal length: width ratio (Figure 3.12) between taxa; this was significant because these measures relate to shape rather than size. The leaf-base angle (Figure 3.9) was significantly smaller on *C. atlantica* plants than on *C. officinalis* subsp. *scotica* or *C. officinalis* s.s plants. This difference was particularly pronounced in population 4 (Fort William, the type location). The mean average measurements for leaves and petals in each taxon (Tables 3.3 & 3.4) echoed the trends seen in the box-plots. *C. officinalis* subsp. *scotica* tended to have shorter petals and leaves than the other taxa; *C. atlantica* leaves tended to have a smaller leaf-base angle. The variance in morphological characters between individuals in populations and between populations was so great that the mean average measurements (Tables 3.3 & 3.4) do not reflect typical measurements for each taxon.

The ANOVA of the morphological characters (Table 3.5) showed that all characters vary significantly between populations. Some morphological variables also varied significantly between taxonomic groups. If we use 95% confidence limits as a guide to significant variation then leaf length, leaf base angle, petal length and petal width all showed significant variation between taxa. The variation between taxa for ratio data (length:width of petals and leaves) was very low, and not statistically significant. The predominant source of variation in morphological characters was between individuals within populations (Table 3.6). The exceptions are petal length and leaf-base angle, where most of the variation was found between taxa. In addition, taxon accounted for 31.1% of the variation in petal width and 27.3% of the variation in leaf length (Table 3.6).

Principal components analysis of the character data (Figure 3.13) revealed that when all of the characters were combined, they could not be used separate the taxa. The ANOVA analysis of the variation in PCA scores (Table 3.7) between groups showed weakly significant variation between taxa and highly significant variation between populations.

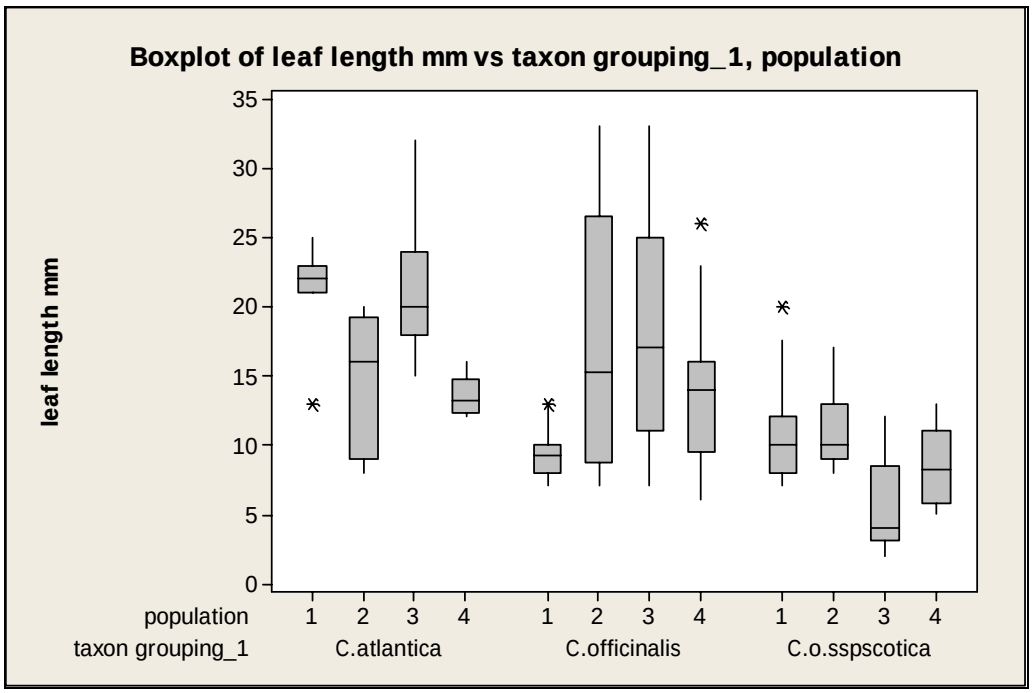

Figure 3.6: Box-plots showing the variation in leaf length (mm) among coastal *Cochlearia* taxa and populations. Order of populations: *C. atlantica*: 1 Dyfi estuary, 2 Oban, 3 Brora, 4 Fort William; *C. officinalis* s.s.: 1 Crammond Island, 2 Sand Bay, 3 Ramasaig, 4 Port Gheiraha O; *C. officinalis* subsp. *scotica*: 1Tain, 2 Chanonary Point, 3 Port Gheiraha S, 4 Kerrara. * = outlier

.

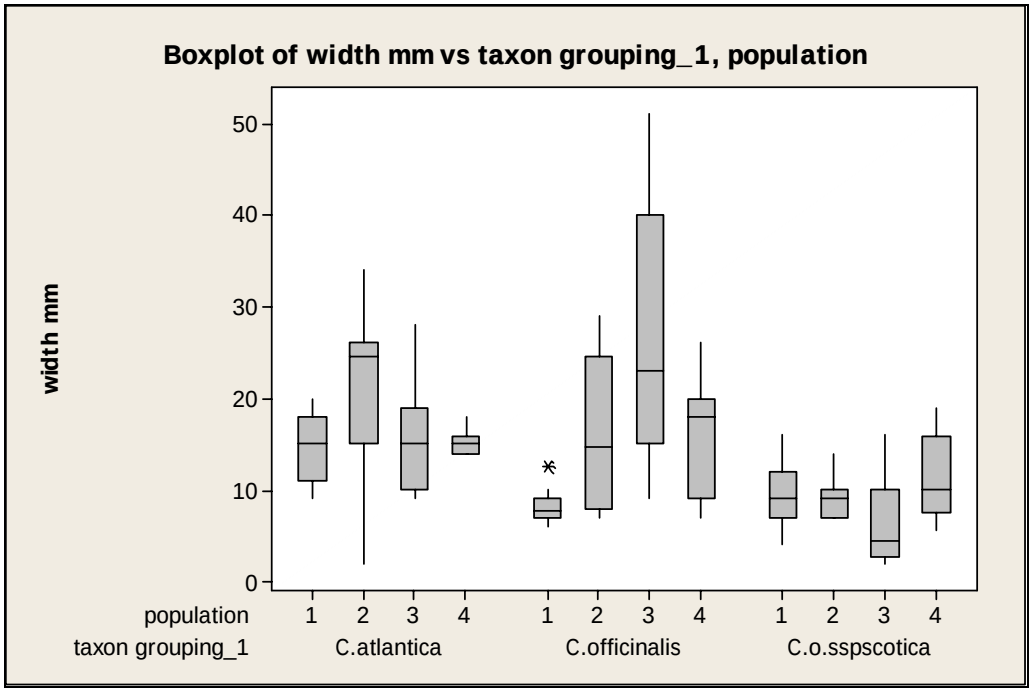

Figure 3.7: Box-plots showing the variation in leaf width (mm) among coastal *Cochlearia* taxa and populations. Order of populations: *C. atlantica*: 1 Dyfi estuary, 2 Oban, 3 Brora, 4 Fort William; *C. officinalis* s.s.: 1 Crammond Island, 2 Sand Bay, 3 Ramasaig, 4 Port Gheiraha O; *C. officinalis* subsp. *scotica*: 1Tain, 2 Chanonary Point, 3 Port Gheiraha S, 4 Kerrara. * = outlier

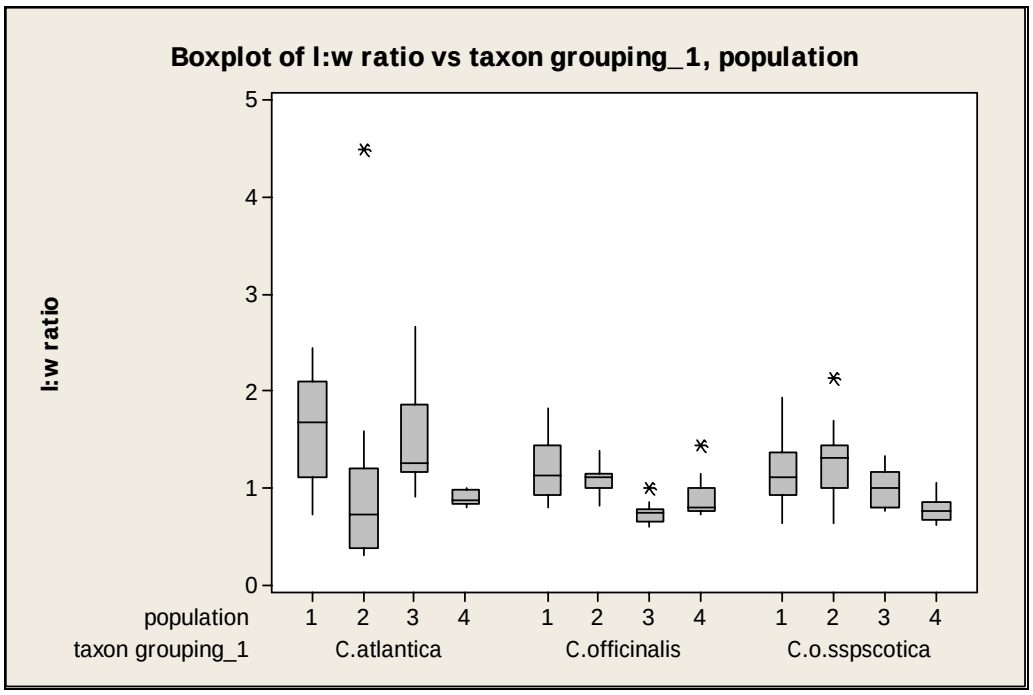

Figure 3.8: Box-plots showing the variation in leaf length:width among coastal *Cochlearia* taxa and populations. Order of populations: *C. atlantica*: 1 Dyfi estuary, 2 Oban, 3 Brora, 4 Fort William; *C. officinalis* s.s.: 1 Crammond Island, 2 Sand Bay, 3 Ramasaig, 4 Port Gheiraha O; *C. officinalis* subsp. *scotica*: 1Tain, 2 Chanonary Point, 3 Port Gheiraha S, 4 Kerrara. * = outlier

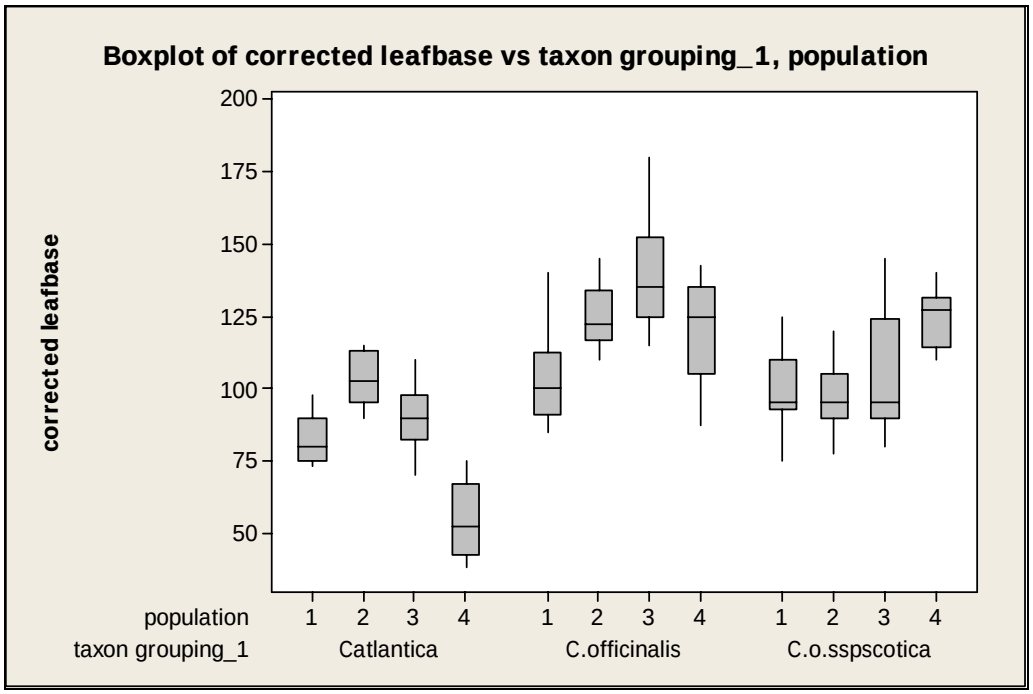

Figure 3.9: Box-plots showing the variation in leaf base angle among coastal *Cochlearia* taxa and populations. Order of populations: *C. atlantica*: 1 Dyfi estuary, 2 Oban, 3 Brora, 4 Fort William; *C. officinalis* s.s.: 1 Crammond Island, 2 Sand Bay, 3 Ramasaig, 4 Port Gheiraha O; *C. officinalis* subsp. *scotica*: 1Tain, 2 Chanonary Point, 3 Port Gheiraha S, 4 Kerrara. * = outlier

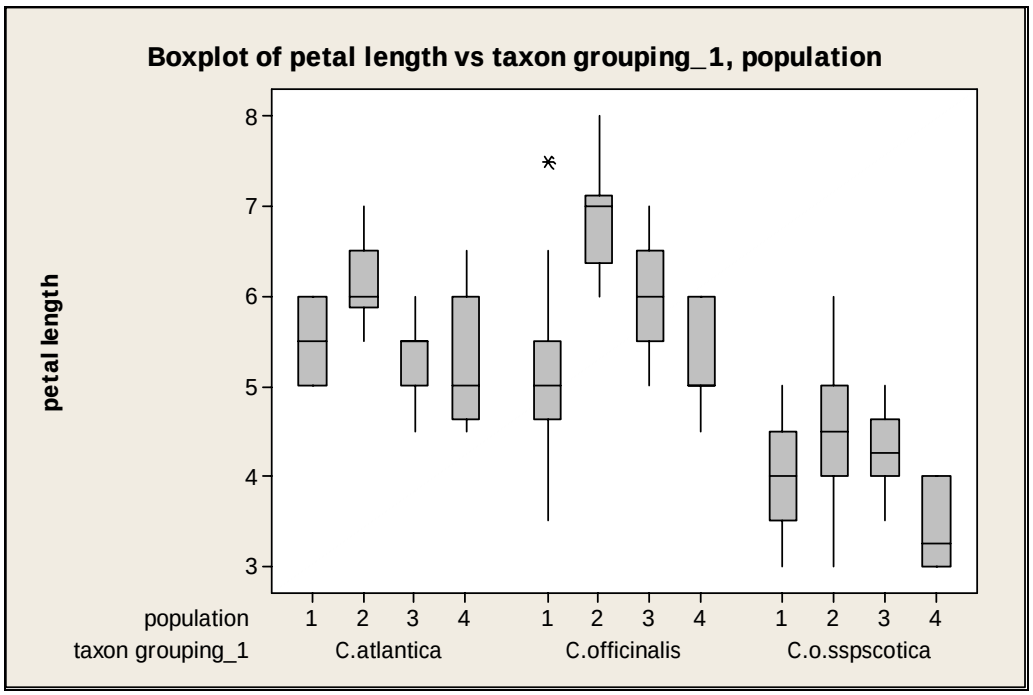

Figure 3.10: Box-plots showing the variation in petal length (mm) among coastal *Cochlearia* taxa and populations. Order of populations: *C. atlantica*: 1 Dyfi estuary, 2 Oban, 3 Brora, 4 Fort William; *C. officinalis* s.s.: 1 Crammond Island, 2 Sand Bay, 3 Ramasaig, 4 Port Gheiraha O; *C. officinalis* subsp. *scotica*: 1Tain, 2 Chanonary Point, 3 Port Gheiraha S, 4 Kerrara. * = outlier

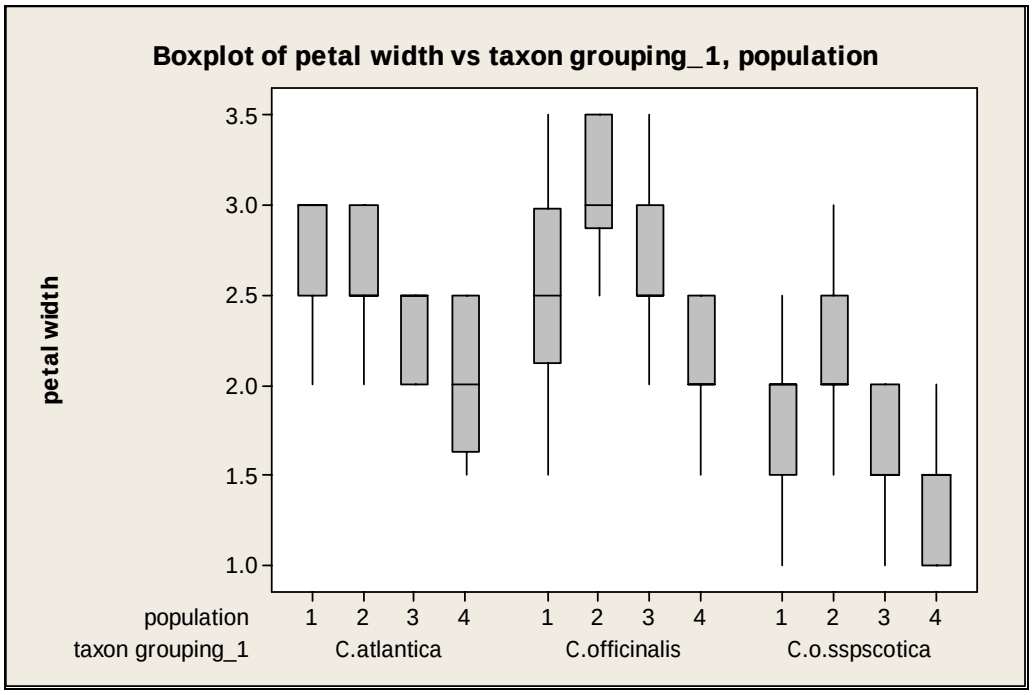

Figure 3.11: Box-plots showing the variation in petal width (mm) among coastal *Cochlearia* taxa and populations. Order of populations: *C. atlantica*: 1 Dyfi estuary, 2 Oban, 3 Brora, 4 Fort William; *C. officinalis* s.s.: 1 Crammond Island, 2 Sand Bay, 3 Ramasaig, 4 Port Gheiraha O; *C. officinalis* subsp. *scotica*: 1Tain, 2 Chanonary Point, 3 Port Gheiraha S, 4 Kerrara. * = outlier

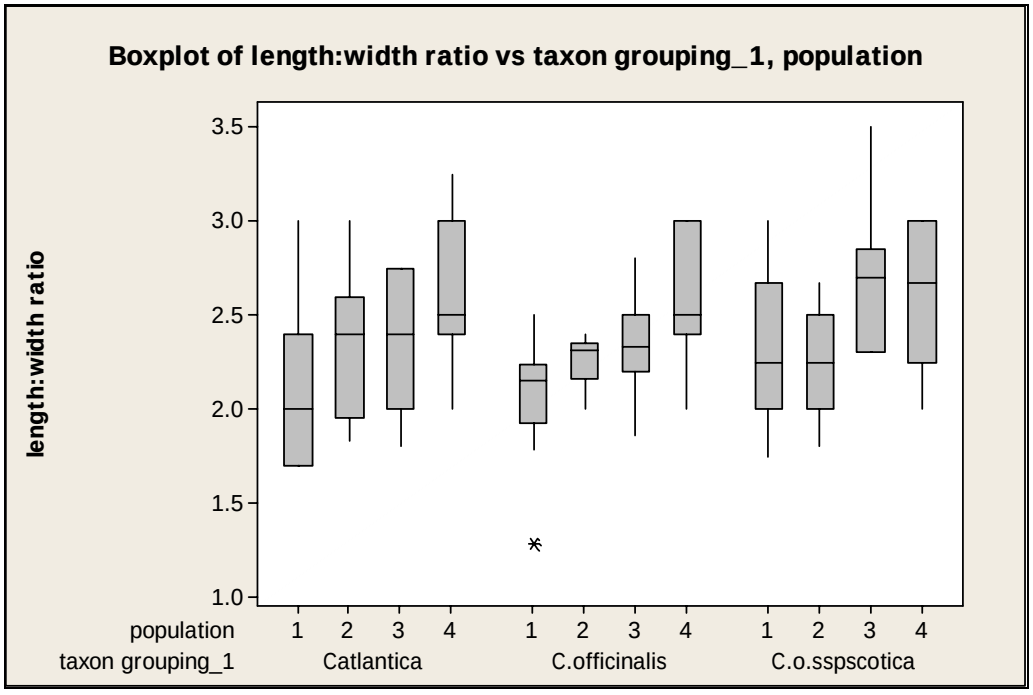

Figure 3.12: Box-plots showing the variation in petal length:width among coastal *Cochlearia* taxa and populations. Order of populations: *C. atlantica*: 1 Dyfi estuary, 2 Oban, 3 Brora, 4 Fort William; *C. officinalis* s.s.: 1 Crammond Island, 2 Sand Bay, 3 Ramasaig, 4 Port Gheiraha O; *C. officinalis* subsp. *scotica*: 1Tain, 2 Chanonary Point, 3 Port Gheiraha S, 4 Kerrara. * = outlier

Taxon	Leaf length (mm)	Leaf width (mm)	Leaf-base angle	Leaf length:width
***C. officinalis* s.s.**	15.1 (±7.8)	17.5 (±11.4)	121.7 (±21.2)	0.9 (±0.3)
C. officinalis* subsp. *scotica	9.4 (±3.8)	9.2 (±3.9)	77.9 (±22.3)	1.1 (±0.4)
C. atlantica	17.8 (±5.4)	16.7 (±6.2)	92.6 (±22.4)	1.3 (±0.7)

Table 3.3: Table of mean average and standard deviation of leaf measurements for each coastal *Cochlearia* taxon

Taxon	Petal length (mm)	Petal width (mm)	Petal length:width
***C. officinalis* s.s.**	5.8 (± 0.1)	2.5 (±0.5)	2.3 (± 0.3)
C. officinalis* subsp. *scotica	4.1(±0.7)	1.8 (±0.5)	2.4 (±0.4)
C. atlantica	5.6 (±0.6)	2.4 (±0.4)	2.4 (±0.4)

Table 3.4: Table of mean average and standard deviation of petal measurements for each coastal *Cochlearia* taxon.

Source of variation		Leaf-base angle	Leaf length	Leaf width	Leaf length: width	Petal length	Petal width	Petal length: width
	DF	MS	MS	MS	MS	MS	MS	MS
Taxa	2	16553.00*	819.78*	911.83	0.89	43.16**	9.83*	0.26
Populations	9	2878.30***	154.62***	318.99***	0.66***	3.60***	1.33***	0.54***
Error	130	213.10	27.00	45.83	0.20	0.40	0.15	0.12
Total	141							

Table 3.5: General linear model ANOVA results for each morphological variable among populations and taxa of Coastal *Cochlearia*. The asterisks represent the P-value level of significance for the mean square result concerned (* = P value < 0.05, ** = P value < 0.01, *** = P value 0.001)

Source of variation	Leaf base angle %	Leaf length %	Leaf width %	Leaf length:width %	Petal length %	Petal width %	Petal length: width %
Taxa	39.4	27.3	15.5	1.8	55.3	31.1	0
Populations	33.6	22.3	30.9	14.8	19.0	18.4	25.3
Individuals	27.0	50.5	53.6	83.5	25.7	54.0	74.7

Table 3.6: The % of variation for each morphological measurement accounted for by taxon, population and individuals, among populations and taxa of Coastal *Cochlearia*

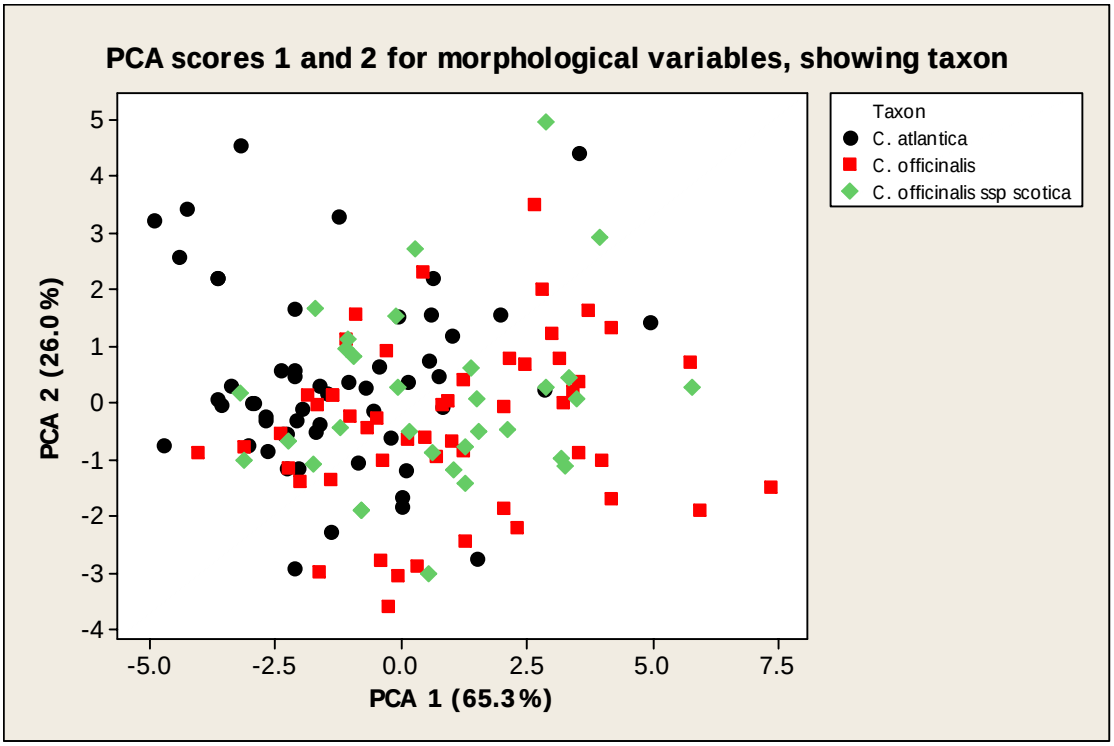

Figure 3.13: PCA ordination based on morphological field measured characters for three coastal *Cochlearia* taxa.

Source of variation	DF	MS
Taxon	2	62.23
Between populations within taxa	9	21.71***
Error	127	4.05
Total	138	

Table 3.7: ANOVA of first principal component score by taxa and populations, based on field characters for three coastal *Cochlearia* taxa. (* = P value < 0.05, ** = P value < 0.01, *** = P value 0.001)

3.3.2 Results of genetic analysis

3.3.2.1 Marker frequency analysis

The AFLP marker analysis produced 242 polymorphic, scorable fragments. The Port Gheiraha population produced the smallest average number of fragments per sample with 36.0. The Chanonary Point population produced the largest average number of fragments per sample with 51.2. There were no diagnostic fragments for any of the taxa or regions (Table 3.8). Only one population had a diagnostic marker, Crammond Island. Crammond Island also had another high frequency private fragment, and Brora had two. The ANOVA of marker number did not reveal significant variation between taxa or regions (Tables 3.9 & 3.10). However, significant variation in marker number was found among populations nested either within regions or within taxa.

3.3.1.2 Analysis of genetic variation based on AFLP markers

The principal co-ordinates analysis based on AFLP marker variation is shown with population highlighted (Figure 3.14) and taxon highlighted (Figure 3.15). The amount of variation accounted for by the first two axes was very low, 7.8% for the first axis and 5.4% for the second. Figure 3.14 shows that individuals from the same populations consistently grouped together in relation to individuals from other populations. Nonetheless there was a great deal of overlap between the different populations. The PCO plot highlighting taxa (Figure 3.15) shows that plants do not group with other members of the same taxon from other populations. Thus AFLP marker variation was not related to taxonomic classification. In the AMOVA analysis of AFLP variation (Tables 3.11 & 3.12), neither region nor taxon explains a significant proportion of the variation. Most of the variation (~80%) was between individuals in both analyses, but a significant proportion of the variation (~20%) was found between different populations.

	Mean number of Fragments	No. fragments polymorphic above the 5% level.	No. private fragments	No. diagnostic fragments	Private fragments present in >50% of population	Private fragments present in one sample (singletons)	% Singleton Private fragments
C. atlantica							
Dyfi Estuary	43.3	45.0	9	0	0	3	60
Oban	37.9	38.4	2	0	0	2	100
Brora	48.3	43.8	6	0	2	2	33
Fort William	42.3	45.5	5	0	0	3	33
Taxon level	42.9		21	0	0	13	62
***C. officinalis* s.s.**							
Crammond Island	40.9	39.9	3	1	2	1	33
Sand Bay	43.6	36.8	2	0	0	2	100
Ramasaig	45.6	40.9	3	0	0	3	100
PortGheirahaO	32.4	36.0	6	0	0	4	67
Taxon Level	40.7		15	0	0	8	53
C. officinalis* subsp. *scotica							
Tain	42.5	43.4	0	0	0	0	0
Chanonary Point	49.4	51.2	3	0	0	3	100
PortGheirahas	45.9	45.5	3	0	0	2	67
Kerrara	46.5	49.2	3	0	0	2	67
Taxon level	46.1		18	0	0	8	44

Table 3.8: The mean number of AFLP fragments, polymorphic fragments, private AFLP fragments and their distribution derived from taxa and populations of three coastal *Cochlearia* species.

Source	DF	MS
Taxon	2	238.81
Between populations within taxa	9	147.66*
Error	82	64.00
Total	93	

Table 3.9: GLM nested ANOVA of AFLP marker number between three coastal *Cochlearia* taxa, with populations nested within taxa. (* = P value < 0.05, ** = P value < 0.01, *** = P value 0.001).

Source	DF	MS
Region	3	83.93
Between populations within regions	8	195.16**
Error	82	64
Total	93	

Table 3.10: GLM nested ANOVA of AFLP marker number between three coastal *Cochlearia* taxa, with populations nested within region. (* = P value < 0.05, ** = P value < 0.01, *** = P value 0.001).

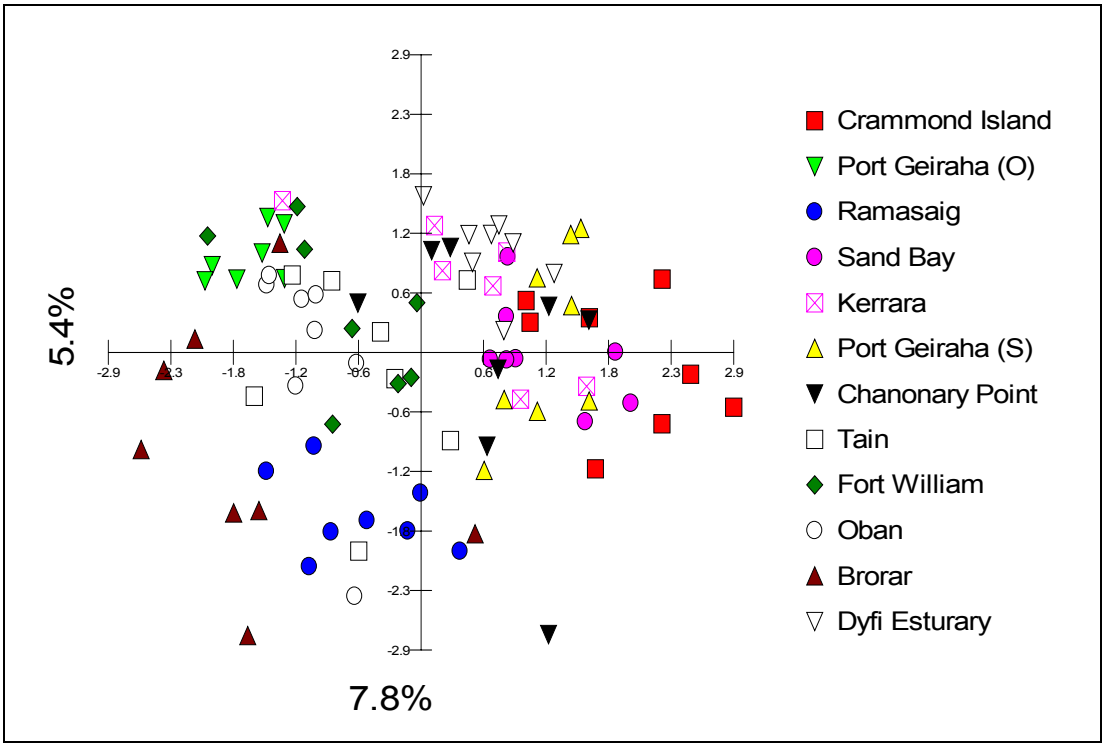

Figure 3.14: PCO plot showing the phenetic relationships between different populations of three *Cochlearia* taxa based on AFLP data converted to Jaccard's similarity coefficients.

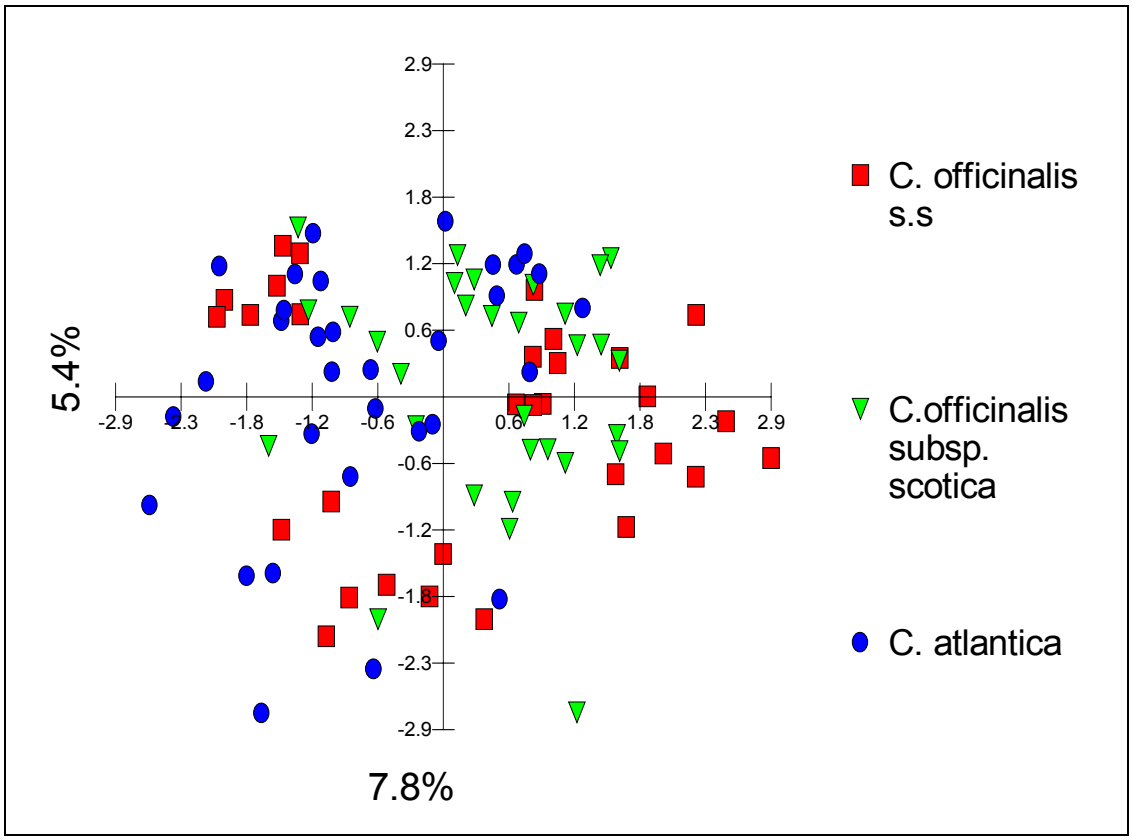

Figure 3.15: PCO plot showing the phenetic relationship among three coastal *Cochlearia* taxa based on AFLP data converted to Jaccard's similarity coefficients.

Source of variation	D.F	Sum of squares	Variance	% of variation	P-values
Among taxa	2	100.37	0.074	0.36	0.33
Among populations within taxa	9	430.98	3.98	19.20	0.00
Within populations	82	1366.50	16.66	80.44	

Table 3.11: AMOVA of AFLP variation among three coastal *Cochlearia* taxa, among populations within taxa.

Source of variation	D.F	Sum of squares	Variance	% of variation	P-values
Among regions	3	131.16	-0.2065	-1.26	0.75
Among populations within regions	8	399.51	4.2407	20.54	0.00
Within populations	82	1366.50	16.6646	80.72	

Table 3.12: AMOVA of AFLP variation of three coastal *Cochlearia* taxa among regions, among populations within regions.

3.3.1.3 Pairwise Φst values and Mantel test

The average Φst value between populations was Φst = 0.195. Pairwise estimates of Φst values derived from population differentiation are given in Table 3.13. Most surprising was the high Φst value between the two sympatric populations at Port Gheiraha (Φst = 0.262). Crammond Island and Port GheirahaO both had high average population pairwise Φst (Φst = 0.262, Φst = 0.247 respectively) values compared with other populations. There were also some very low Φst values, particularly for pairwise estimates involving Tain and Port Gheiraha that do not appear to be related to geography or taxonomy. When these genetic distances between populations were compared with geographical distances using a Mantel test there was no significant relationship ($r^2 = 0.02$).

	CramIsO	PortgeO	RamasaigO	SandbayO	KerraraS	PortGS	chanpoiS	TainS	Fort WilliamA	ObanA	BroraA	DyfiestA
CramIsO	0.000											
PortgeO	0.363	0.000										
RamasO	0.292	0.267	0.000									
SandbaO	0.274	0.293	0.266	0.000								
KerraraS	0.195	0.203	0.169	0.136	0.000							
PortGS	0.178	0.262	0.227	0.074	0.084	0.000						
ChanpoiS	0.199	0.264	0.210	0.190	0.115	0.130	0.000					
TainS	0.245	0.211	0.160	0.217	0.136	0.144	0.100	0.000				
Fort WilliamA	0.262	0.155	0.214	0.198	0.116	0.146	0.136	0.072	0.000			
ObanA	0.305	0.188	0.169	0.253	0.149	0.171	0.153	0.097	0.079	0.000		
BroraA	0.356	0.246	0.193	0.287	0.243	0.235	0.219	0.183	0.174	0.165	0.000	
DyfiestA	0.217	0.271	0.238	0.202	0.104	0.095	0.139	0.149	0.159	0.170	0.250	0.000
Mean pop. average	0.262	0.247	0.219	0.217	0.150	0.159	0.169	0.156	0.156	0.173	0.231	0.181

Table 3.13: Pairwise Φst estimates between populations of three coastal *Cochlearia* taxa, *C. officinalis* marked with O, *C. officinalis* subsp. scotica marked with S, *C. atlantica* marked with an A. All Φst values significant to P = 0.05 level.

3.3.1.4 Analyses of the sympatric populations at Port Gheiraha

Of the 59 fragments shared between the two populations only three fragments were not present in *C. atlantica* populations as well (Table 3.14). The shared fragments also showed a strong tendency towards high frequency, 34/59 fragments are present in more than 25% of the samples and 19/59 fragments are present in more than 50% of the samples (Table 3.14). Nineteen of the fragments present only in the *C. officinalis* subsp. *scotica* population and absent in *C. officinalis* population were common occurrence markers (occur in more than 50% of samples) in the *C. officinalis* subsp. *scotica* population. *C. officinalis* subsp. *scotica* had almost as many fragments that were not shared as those that were shared. If the populations had a recent common origin or there was gene flow going on between these two populations, commonly occurring fragments in one population would be expected to appear in the other population.

Taxon	**Private fragments (>50% samples)**	**Shared fragments**
***C. officinalis* s.s. (PortGheirahaO)**	32 (0)	59
***C. officinalis* subsp. *scotica* (PortGheirahaS)**	56 (19)	

Table 3.14: AFLP fragment frequency analysis for between two sympatric populations at Port Gheiraha

The PCO plot with the two Port Gheiraha populations highlighted (Figure 3.16) showed that the two populations at Port Gheiraha were genetically differentiated from each other in comparison with the other coastal *Cochlearia* populations. The evidence from the PCO plot was supported by the AMOVA results between the population pairs, which attribute over 26% of the total variation to variation between the two populations (Table 3.12)

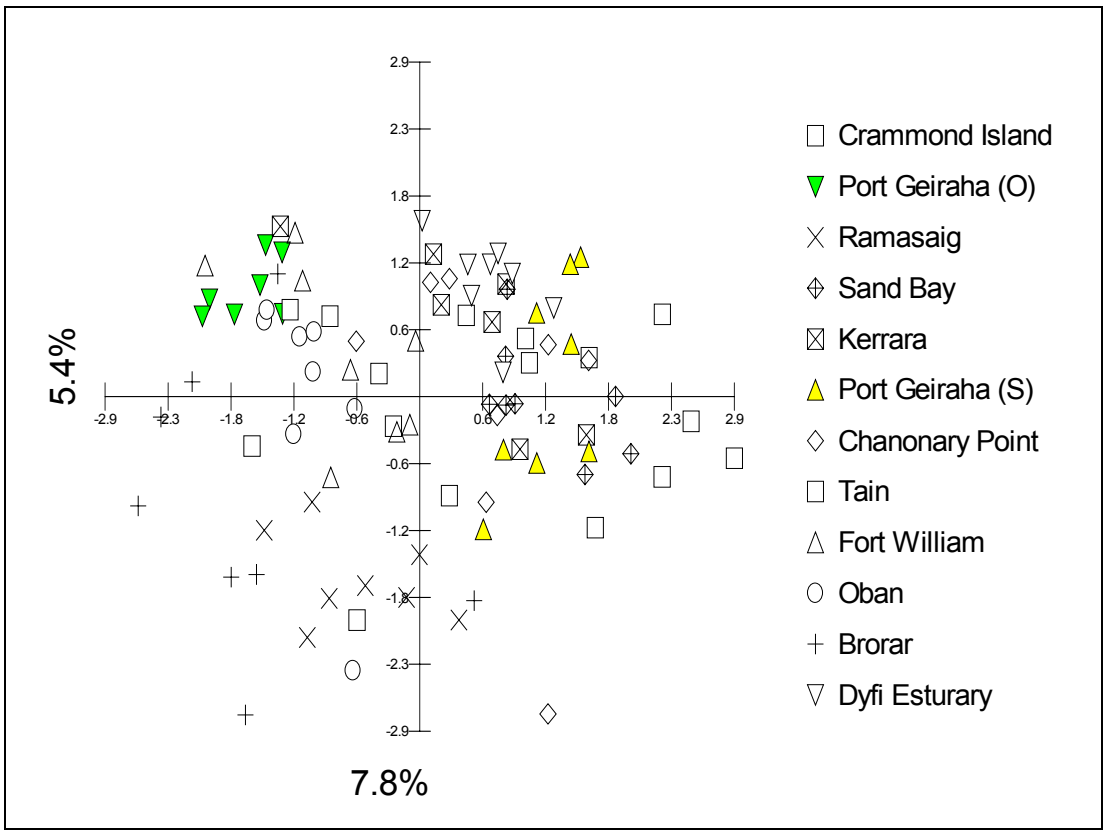

Figure 3.16: PCO plot showing the phenetic relationship between the two populations at Port Gheiraha compared with the variation among all the coastal populations, based on AFLP data converted to Jaccard's similarity coefficients.

Source of variation	D.F	Sum of squares	Variance	% of variation	P-values
Among populations	1	61.21	5.9562	26.25	>0.0001
Within populations	13	217.59	16.7376	73.75	
Total	14	278.80	22.6939		

Table 3.12: AMOVA of AFLP variation among the two sympatric populations at Port Gheiraha.

3.4 Discussion

3.4.1 Discussion of morphological results

The persistence of population-specific morphological characters in cultivation suggests that some morphological variation has a genetic basis. There is also some evidence for phenotypic plasticity between the cultivated and wild populations. Consideration of the data from cultivated and wild populations leads to the following conclusions: a) there is significant morphological variation between populations and high levels of variation among individuals; b) there is significant variation for some morphological characters between taxa; c) the statistics for length: width ratios do not vary significantly; d) the only character which varies between taxa that is not related to size is leaf-base angle; e) the morphological characters combined do not form groups of characters that can effectively identify taxonomic groups.

The lack of differentiation between taxa for the ratio data shows that there are proportional changes in size between different taxa, rather than changes in shape. Size related characters are less reliable as a proxy for underlying genetic differences, because plant size can vary according to environmental conditions, for example, nutrient levels. Plants adapted to low nutrients may also be small morphs, without significant evolutionary change having occurred (Nordal *et al.* 1989)

It should be stressed that some significant morphological differences between taxa for individual characters are expected because these same characters were used in the selection of samples to represent each taxon. The sampled populations were not a random sample of the overall variation. However, while individual characters do vary between taxonomic groups, the PCA plot shows that, when used together, the morphological characters do not define mutually exclusive groupings of individuals that correspond to named taxa. Thus there was no evidence of correlated discontinuities in the distribution of the morphological characters. Morphological differences between plants in different habitats and populations are likely to be a mixture of adaptive genetic differences and a plastic response to environmental conditions.

3.4.2 Discussion of genetic analysis results

The lack of diagnostic fragments among populations, taxa or regions suggests that populations and higher level groupings have not been genetically isolated from each other

for a long period of time, if at all. The number of fragments varies significantly between different populations, but not between different taxa or regions. The causes of variation in marker number between different populations of *Cochlearia* are unknown.

As with the morphological data, a considerable proportion of the observed variation can be attributed to between population variation. Neither the ordination analyses, nor the AMOVA analyses reveal any taxonomic signal in the data. Furthermore, the PCO plot highlighting taxa showed that plants with the same taxonomic identification do not group together. The between population differentiation was not generally equivalent to the geographical distance between them. The highest pairwise Φst values were found for the populations at Dyfi estuary and Crammond Island, which were both geographically isolated from other populations. Other pairwise Φst values do not seem to relate to the distance between sampled populations e.g. Tain (E. Scotland) and Oban (S. W. Scotland) had a pairwise Φst of Φst = 0.097.

3.4.3 Post glacial colonisation scenarios

There are two main scenarios that could have given rise to these data. Firstly, the individual taxa could, at one time, have been distinct, and subsequent gene flow among them has led to a merging of their gene pools and also a disruption of any correlation between genetic and geographical distances. Given that much of the current British range was under ice in the last glacial maxima C.18 000 years before present, it is unlikely that all populations would have re-converged in such a uniform way. Recent colonisation from a common source or closely related sources appears to be a more parsimonious explanation. The theory of recent colonisation and limited divergence is supported by the very low chloroplast variation among the populations across Europe (Koch *et al.* 1996). The recent spread of *Cochlearia* into habitats in Britain means that there will be many ancestral similarities in the genome even where there is no gene flow. Different taxa are probably polytopic ecotypes that have arisen independently in different places. The reasonably high θst values suggest fairly low gene flow between populations which would help to maintain these local differences.

3.4.4 Differentiation between sympatric populations at Port Gheiraha

Strong evidence for local differentiation was found between the two populations at Port Gheiraha. The two populations are located only 200 metres apart and show large morphological differences. The results of the genetic analysis show that there is also considerable AFLP marker differentiation between them. The analysis of shared and private

fragments between the populations at Port Gheiraha, suggests that there has been no gene flow from the *C. officinalis* subsp. *scotica* population, to the *C. officinalis* population. It is extremely unlikely that the eighteen high frequency private fragments in *C. officinalis* subsp. *scotica* would not have moved across the other population if gene flow had occurred. The *C. officinalis* population (Port GehariaO) also had private fragments, but all at low frequency, again indicating lack of gene flow between the populations.

The cause of the apparent reproductive isolation between the two populations at Port Gheiraha is unknown. There may be a barrier to gene flow between the two populations. If there is a barrier to gene flow the mechanism that causes it is unclear. The plants were both flowering at the same time, so a temporal shift in flowering cannot be implicated. The *C. officinalis* subsp. *scotica* plants are small and inconspicuous and possibly not very attractive to pollinators. It is possible that there has been a partial shift in breeding strategies towards inbreeding in this population (Wendt *et al.* 2002). Secondly, in the absence of intermediate habitat, strong selection in each habitat can reduce survivorship of non-adapted genotypes, creating strong differentiation between adult populations even where there is gene flow (Linhart & Grant 1996). A third possibility that reproductive isolation is not a factor in the differentiation between the two populations, but that one population has recently colonised from elsewhere and shows high residual differentiation.

The data do not allow us to distinguish between sympatric divergence and secondary contact after divergence in allopatry. However, if sympatric divergence was responsible, one would not expect such high differentiation between the two sub-populations (Φst = 0.26) and (Figure 3.16). The high Φst is most compatible with the allopatric differentiation and secondary contact scenario. Port GheirahaO had high pairwise Φst values (Table 3.13), which may be because it has been isolated for a long time. Port GheirahaS had low pairwise Φst values with populations that grow much further south. This result, combined with the high Φst between this population and its sympatric population, indicate that the Port Gheiraha *C. officinalis* subsp. *scotica* population had recently colonised from somewhere else.

3.4.5 Is there evidence for any distinct coastal endemic *Cochlearia* taxa in Scotland?

There is no evidence for an independent evolutionary lineage of ‘atlantica-type’ plants. The distribution of this putative taxon is unclear. A morphological type with truncate-based

leaves was found, but this type did not constitute a neutral genetic grouping. The difference in the leaf-base angle of *C. atlantica* compared with *C. officinalis* subsp. *scotica* and *C. officinalis* s.s. was striking (particularly at the type locality). The cause of this difference in leaf shape is unknown. Environmental plasticity seems unlikely as the habitats in which three taxa are found are so similar. The leaf shape was also maintained in cultivation. In the absence of neutral genetic differentiation, the most likely explanation is that minor genetic changes that influence leaf shape have created a variant with the typical *C. atlantica* leaves. This is supported by research on *Capsella bursa-pastoris* (Shull 1918, 1929), which sowed that the shape and lobing of rosette leaves was controlled by only two alleles.

There is no evidence that *C. officinalis* subsp. *scotica* represents an independent evolutionary lineage. There were small-leaved, compact plants, which maintained the same form in cultivation under standard conditions. This small ecotype could arise as a result of low nutrient conditions as described in the Scandinavian *Cochlearia* (Nordal *et al.*1986). Many of the scotica sites were also grazed, which could elicit a change to a low growth form (Díaz *et al.* 2007). This trait could be very strongly selected, because plants with tall inflorescences will be eaten before they can flower or set seed. Shingle plants may also respond to wave action and substrate movement with a low spreading growth form. Although these plants may have a different morphology, they do not necessarily signify the presence of evolutionary lineages. Coastal environmental heterogeneity has provided a range of niches to which *Cochlearia* have colonised and adapted to. This process has not been accompanied by major genetic change, but by small environmental adaptations or intrinsic developmental plasticity. If there were profound changes in the genome, we would expect a fixed suite of morphological characters and perhaps a greater degree of reproductive isolation.

3.5 Conclusions

No evidence was found that neither *C. officinalis* subsp. *scotica* nor *C. atlantica* types represent distinct coastal endemic *Cochlearia* species in Scotland. There is no evidence for established evolutionary lineages that we might call species among the Scottish coastal *Cochlearia*. The array of adaptive morphologies has lead to the classification of three species, which roughly correspond to adaptive ecotypes. Ecotypes were given subspecies classifications among the Scandinavian *Cochlearia* (Nordal & Laane 1990), but the problem of distinguishing the subspecies remains unaddressed. Species-based conservation of these *Cochlearia* taxa is not appropriate because we cannot delimit them as species. However,

complex species aggregates, such as *Cochlearia* form a considerable part of Scottish biodiversity. The conservation of morphological and ecological variation is therefore recommended, by the protection of a range of coastal habitats that contain different types of *Cochlearia.*

4. Morphological and genetic variability in the Scottish upland *Cochlearia*, focussing on the putative endemic *C. micacea*

Abstract

The aim of this chapter was to investigate whether the putative Scottish endemic *Cochlearia* species *C. micacea* formed a discrete genetic and morphological group when compared with the two other upland taxa: *C. pyrenaica* subsp. *alpina* and *C. pyrenaica* subsp. *pyrenaica*. Upland populations of the *Cochlearia officinalis* s.l. complex were sampled with particular attention to the type location of *C. micacea* and other putative populations of this taxon. The collected samples were then screened for leaf characters and AFLP marker variation. Pod characters and genetic similarity were also compared for the plants from the *C. micacea* type location. *C. micacea* did not form a discrete genetic grouping compared with the other upland populations. Therefore *C. micacea* does not appear to be a distinct, endemic species.

4.1 Introduction

4.1.1 Upland *Cochlearia* species in Britain

There are three named *Cochlearia* taxa in the British uplands, *C. pyrenaica* DC. subsp. *alpina* (Bab.) Dalby, *C. pyrenaica* DC. subsp. *pyrenaica*, and *C. micacea* E. S. Marshall. These taxa are generally considered distinct from the taxa that inhabit the coast. However, there has been a great deal of fluctuation in the classification of *Cochlearia*, with the taxonomy undergoing regular changes (see Table 1.1, Chapter 1). *Cochlearia micacea* is considered to be a rare endemic species and has a UK Biodiversity Action Plan, requiring conservation management and monitoring of populations. However, surveying this species is problematic, because of the number of populations of uncertain taxonomic status. Taxonomic difficulties have created a barrier to conservation management, as it is not known what populations should be conserved, if any. As with the coastal *Cochlearia* taxa, the taxonomy of this species must be clarified before conservation management can proceed.

4.1.2 Upland British *Cochlearia* in the European context

Upland populations of *Cochlearia* occur in all the major mountain ranges of Europe. The diploid *C. pyrenaica* subsp. *pyrenaica* which is found in Northern England and on Skye is also widespread in the European mountain ranges. The tetraploid *C. pyrenaica* subsp. *alpina*

is not present in the rest of Europe (Koch 2002). *C. micacea* may be synonymous with the inland, tetraploid *C. officinalis* subsp. *integrifolia* of Norway and therefore not endemic to Britain (Nordal & Stabbetorp 1990). The taxonomic confusion in Britain has been mirrored by similar problems in Eastern Europe, with localised taxa being declared species only to be later rejected (Pobedimova 1970, 1971**,** Jalas *et al.* 1996). There, *C. pyrenaica* and *C. officinalis* are considered to have acted as the progenitor species for a number of localised hexaploid taxa (see 1.1.2.2).

4.1.3 Post glacial history.

The diploid populations of Northern England and Skye are thought to be remnants of early colonisers from *C. pyrenaica* populations that persisted in Southern Britain during the last glacial maxima (Godwin 1964, Godwin 1975 in Lang 1995, Gill *et al.* 1978). The diploids are the ancestral types from which the polyploids, including *C. pyrenaica* subsp. *alpina* and *C. micacea* are considered to have been derived (Gill *et al.* 1978, Koch *et al.* 1998).

4.1.4 Taxonomic characters

4.1.4.1 Morphological characters that distinguish the three upland species

Cochlearia officinalis L. subsp. *alpina* Bab. was described with no type specimen (Babington 1843). There has been considerable confusion over which populations and morphological types this name represents (see also section 1.5.15, chapter 1). For the purposes of this thesis *C. pyrenaica* subsp. *pyrenaica* is taken to refer to the diploid (2n = 12) found in Northern England and at one site on Skye. *Cochlearia pyrenaica* subsp. *alpina* refers to the 2n = 24 upland taxon of Scotland and Wales, as described by Dalby (1991). *Cochlearia micacea* was delimited on the grounds of morphological differences (see Figure 4.1) from other upland taxa and the constancy of these characters when grown from seed (Beeby 1889, Marshall 1894). There are a number of characters described to distinguish between the upland *Cochlearia* taxa. At first it appears that there are a good suite of characters to distinguish each taxon (Table 4.1), however, under closer inspection many of the characters are subjective or comparative and thus difficult to interpret. Many confusing populations are encountered in the field

Character	*C. micacea*	*C. pyrenaica* subsp. *alpina*	*C. pyrenaica* subsp. *pyrenaica*
Seed pods	Three times as long as wide, often asymmetric with no veins	Rounded with reticulate veination	Rounded with narrow bases
Roots	Thick, woody	Herbaceous rootstock	Herbaceous rootstock
Basal Leaves	Dark green, shiny leaves, shallowly cordate and fleshy	Dull green leaves, basal leaves often reniform, fleshy	Thin leaves
Plant size	Up to 15cm width, Up to 8cm max height	Up to 20cm, in flushes	Up to 30cm
Petals	Squared petals, long claw	Longer petal, tapering to claw	Longer petal
Growth form	Erect	Decumbent	Erect
Sepals	Dark green	Light green	Light green
Flower colour	White	Typically white, occasionally lilac	White
Stem leaves	Sparse stem leaves, entire, without auricles	Many toothed stem leaves near stem apex	Many stem leaves

Table 4.1: Distinguishing morphological characters between the three upland species: *C. micacea*, *C. pyrenaica* subsp. *alpina*, *C. pyrenaica* subsp. *pyrenaica* (Dalby 1994).

4.1.4.2 Cytological characters that distinguish the three upland species

The publication of chromosome counts for various populations (see Table 1.2, Chapter 1 & Figure 4.1) defined three ploidal numbers that roughly corresponded to the three previously described taxonomic groups. Chromosome counts for *C. micacea* have been recorded as 2n = 26, *C. pyrenaica* subsp *alpina* as 2n = 24 and *C. pyrenaica* subsp pyrenaica 2n = 12 (Gill *et al.* 1978). The chromosome number is one of the strongest delimiting characters for these taxa. However, chromosome number is not a field character, nor does it correspond closely with suites of field characters. There are many morphologically and ecologically ambiguous populations where no chromosome counts have been made.

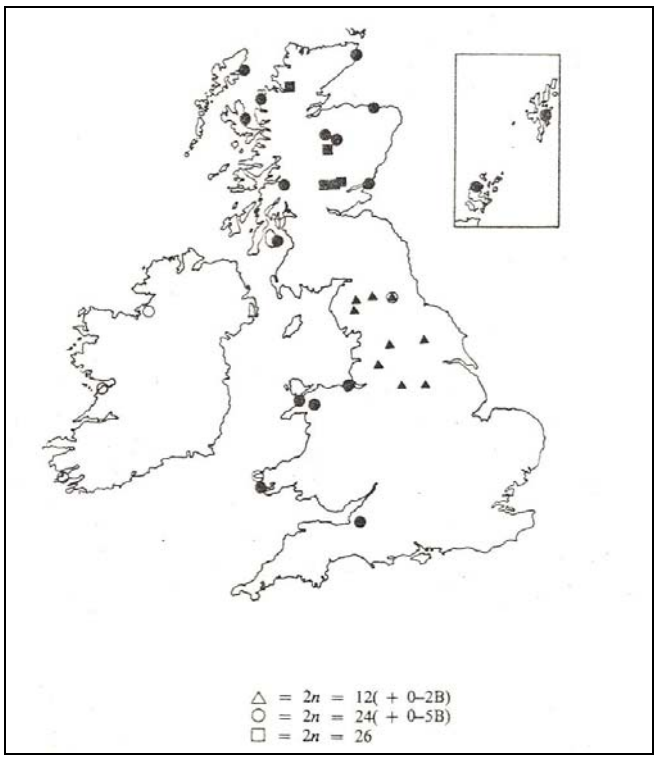

Figure 4.1: Map reproduced from Gill *et al.* (1978), showing the sites of British *Cochlearia* populations from which chromosome counts have been recorded, including the recorded count.

4.1.5 Potential adaptations to upland habitats

The environmental conditions experienced by upland *Cochlearia* species in Britain are very variable, from sheltered, mobile gullies (e.g. Lochnagar), to exposed serpentine sites like Little Kilrannoch and the base-rich river banks and springs favoured by *C. pyrenaica* subsp *pyrenaica* in Northern England. Populations in the uplands may have specific adaptations to different types of upland habitat, as well as adaptations to upland habitats in general. There are often small localised niches within upland habitats that can mean that one mountain population experiences very different conditions to another nearby. An example of this is the difference in conditions on leeward versus the windward side of boulders (Billings 1974). Many of the upland *Cochlearia* populations live in flushes or on ledges with constant running water (pers. obs. 2004, 2005), so while drought is not a problem, plants must tolerate waterlogged roots. *Cochlearia* are evergreen, passing the winter without a dormancy phase. Therefore the vegetative rosettes must withstand extremely cold and windy conditions, as well as snow cover in many places. Adaptations to these conditions have been noted in studies on the *Cochlearia* of the Netherlands and Scandinavia. Flower buds develop earlier in ecotypes that are covered by snow in the winter, and so that they ready to flower when the

snow melts, as an adaptation to short growing season (Nordal & Stabbetorp 1990). Seed germination of *C. pyrenaica* is inhibited by low light levels (Pegtel 1999), which may prevent germination before the snow has melted.

4.1.6 The Ben Lawers population

C. micacea was first described on the basis of plants from Ben Lawers, Ben an Dothaidh and Am Bennein (Marshall 1894). Populations at the site grow in different habitat types. There are populations near the summit of Ben Lawers, among herb-rich alpine flora. In a quite different habitat at a lower altitude, there are plants growing in flushes. In 1993, the total number of *C. micacea* plants on Beinn Ghlas, Ben Lawers and An Stuc in the Ben Lawers National Nature Reserve was estimated as 12 000, making this by far the most important site for *C. micacea* (Clarke 1993). Gill *et al.* (1978) also recorded a chromosome count of 2n = 26 here, which he believed corresponded with the species *C. micacea*.

The author E. S. Marshall recorded *C. micacea* and *C. alpina* (= *C. pyrenaica* subsp *alpina*) at Ben Lawers, corresponding to separate populations of different morphological types. Pod morphology is important for the identification of *C. micacea*. The pods of *C. micacea* should be three times as long as wide. The morphology of plants at Ben Lawers can be very variable within population (pers. obs. 2004, 2005) and this variation includes variation in pod morphology. In many populations pod shape appears to correspond more closely to that described for *C. pyrenaica* subsp. *alpina,* than that described for *C. micacea.*

There are three possibilities for the status of the populations at Ben Lawers a) that there are mixed populations with both *C. pyrenaica* subsp. *alpina* and *C. micacea*, b) that there are different populations of different taxa c) that all the plants at Ben Lawers are of one taxon, either *C. micacea* or *C. pyrenaica* subsp. *alpina*. If *C. micacea* cannot be substantiated as a distinct taxon at Ben Lawers, one of sites mentioned in the original description (Marshall 1894), this would throw serious doubt as to whether the taxon exists at all.

4.1.7 Questions

1) Traditional taxonomic work predicts three genetically distinct groups in the uplands: the diploid *C. pyrenaica* subsp. *pyrenaica*, the tetraploid *C. pyrenaica* subsp *alpina* and the aneuploid *C. micacea*. Are there frequency differences or diagnostic fragments from AFLP analysis that confirm that prediction?

2) Are there differences in leaf morphology that correspond to genetic or taxonomic groupings (as defined above)?

3) Among the Ben Lawers samples, are individuals with a similar pod length more similar genetically?

4) Marshall believed that both *C. pyrenaica* subsp. *alpina* and *C. micacea* were present at the Ben Lawers site, can two distinct groups be found now?

4.1.8 Approach

A range of morphological types were sampled from eleven populations in the uplands of Scotland and Northern England, with particular attention paid to putative *Cochlearia micacea* populations. The Ben Lawers range in Perthshire, Scotland, was chosen as a focus for *C. micacea* sampling and for testing the existence of *C. micacea* as a distinct species. These samples were then measured for four leaf dimensions, which were then analysed using descriptive statistics, PCA and ANOVA. The upland samples were screened for chloroplast DNA polymorphisms using the PCR-RFLP technique. Then the samples were screened for AFLP marker variation and analysed using PCO analysis and AMOVA analysis. The aim was to find whether there were three clusters of variation in any of the data sources that might correspond to taxonomic groups.

4.2 Materials and Methods

4.2.1 Sampling of upland populations

Sample sites were chosen in Scotland and England with particular attention to putative *C. micacea* populations (Figure 4.2). Ten-fifteen plants were sampled from each population, and voucher specimens were taken to represent each population (Table 4.2). A list of associated species was also recorded for each population (Table 4.2). Plants described as *C. micacea* were from populations confirmed as *C. micacea* in a survey in 1994 (Dalby & Rich 1994). All taxa were identified following the descriptions in the Botanical Society for the British Isles 'Crucifer handbook' (Dalby 1991), also with reference to New Flora of the British Isles' (Stace 1997). *C. micacea* has a highly restricted distribution, and therefore it was impossible to choose populations over a broad geographical area.

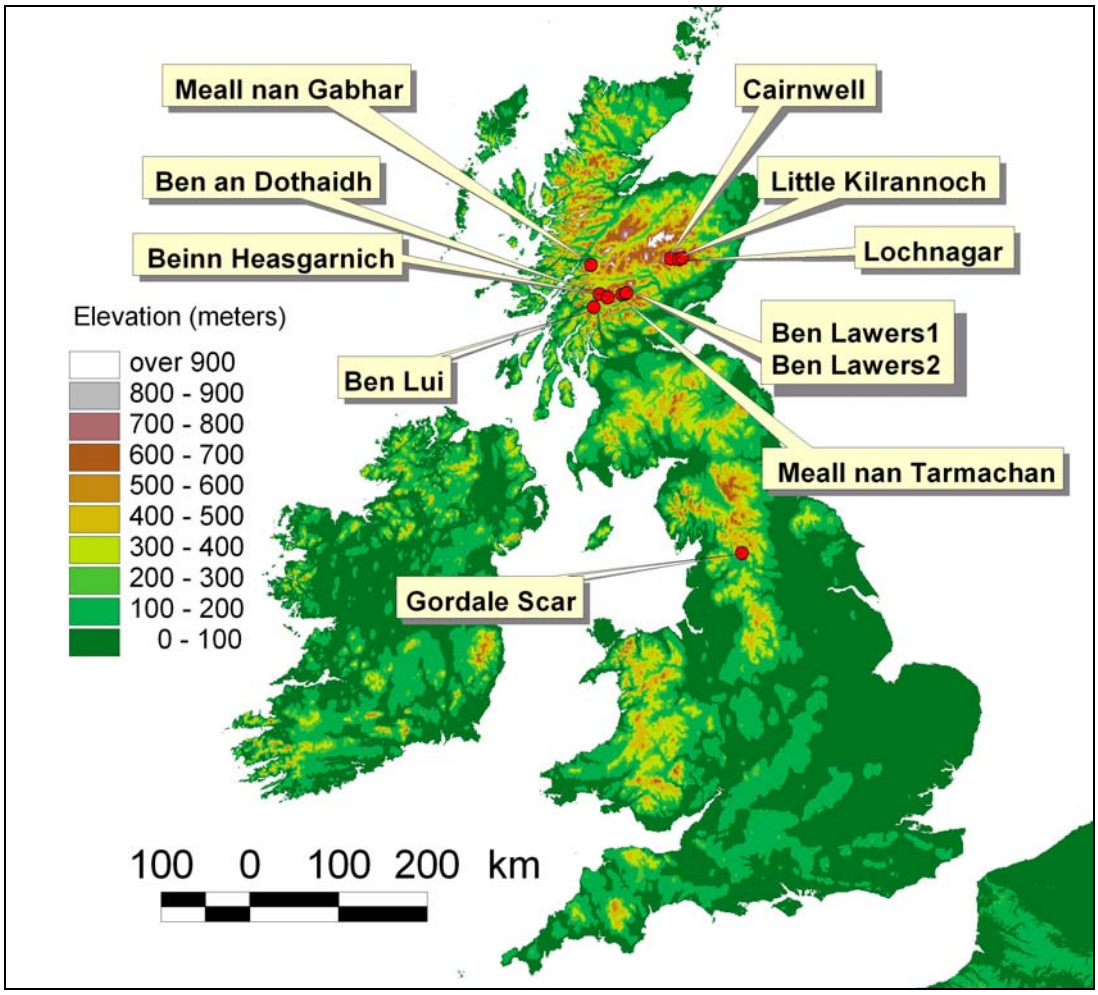

Figure 4.2: A map of Britain showing the location of the ten upland populations of three taxa sampled in order to investigate morphological and genetic variation.

4.2.1.1 Sampling at the Ben Lawers site.

At Ben Lawers plants were sampled from two different sites. One population was near the summit of Ben Lawers from herb-rich, rock ledge community, the other was at a lower altitude flush. In order to link the morphology of the plant with the genetic data, each sampled plant was collected whole. Only plants with seed pods were chosen for sampling and a few leaves were removed and dried in silica gel for genetic analysis.

4.2.2 Greenhouse plants

Plants and seeds were collected from wild populations for a reference collection of different taxa. They were also used to make inferences about the influence of the environmental variables on the study populations. Whenever possible seeds were collected from each population, otherwise live plants were collected. The collections could not be used for formal statistical analysis because there were only small numbers of plants and some were grown from seed, others were collected as adults. The plants and seeds were then cultivated in the glasshouse under standard conditions, with the same compost.

4.2.3 Morphological measurements

Samples were collected to measure leaf characters from all but one of the sampled populations. The number of leaves collected varied according to the population size. The size of the populations in the uplands was generally much smaller than the population size on the coast. The number of flowers produced per plant or population was also much lower. Petals could only be collected in useful numbers from three of the sampled upland populations; therefore the analysis of petal dimensions was not carried out. The length and width of each pod on each sampled plant was measured from the Ben Lawers samples.

Site & Vice County	Putative species	Grid reference	Chromosome counts	Number sampled		Approx. Altitude	Associated species list
				AFLP	Morph		
Ben Lawers, Mid-Perthshire	***C. micacea***	NN/626.407		12	15	900m	*Polygonum viviparum, Alchemilla glabra, Myosotis alpestris, Armeria maritima, Sedum rosea, Silene acaulis, Festuca ovina, Parnassia palustris, Cerastium alpinum, Festuca vivipara, Poa alpina*
Ben Lawers2, Mid-Perthshire	***C. micacea***	NN/633.412	26	11	14	1050m	*Deschampsia cespitosa, Festuca rubra, Festuca ovina, Silene acualis*
Ben an Dothaidh, Main Argyll	***C. micacea***	NN/326 411		7	15	750m	*Saxifraga aizoides, Deschampsia cespitosa, Oxyria digyna, Geranium sylvaticum , Alchemilla glabra, Viola riviniana*
Cairnwell, Angus	*C.p.* subsp. *alpina?*	NO/127.781		8	10	800m	*Festuca ovina, Alchemilla alpina, Sphagnum auriculatum, Anthelia julacea*
Beinn Heasgarnich, Mid Perthshire	***C. micacea***	NN/429.379	26	8	6	950m	*Festuca ovina, Alchemilla glabra, Saxifraga stellaris, Carex echinata, Bryophytes, Saxifraga aizoides, Oxyria digyna, Carex viridula, Taraxacum officinale*
Lochnagar, South Aberdeenshire	*C.p* subsp. *alpina*	NO/246.785	24	8	7	950m	*Saxifraga stellaris, Carex flacca, Saxifraga azoidies, Sedum rosea, Silene acaulis, Poa glauca*
Ben Lui, Mid Perthshire	***C. micacea***	NN/ 265 274		8	15	800m	*Alchemilla glabra, Viola riviniana, Carex capilliaris, Dryas octopetala, Pyrola rotundifolia, Oxyria dygyna, Deschampsia cespitosa, Cardamanopsis petraea*
Meall nan Gabhar, Mid Perthshire	*C. p.* subsp. *alpina*	NN/234.724		8	0	700m	*Alchemilla glabra, Geum rivale, Equisetum arvense, Festuca ovina, Rumex acetosella, Saxifraga stellaris.*
Little Kilrannoch, Angus	*C. p.* subsp. *alpina?*	NO/218.772		6	10	840m	*Festuca rubra, Agrostis stolonifera, Armeria maritima, Cerastium fontanum* subsp. *scoticum, Lychnis alpina, Cherleria sedoides, Sagina subulata, Carex bigelowii, Agrostis vinealis.*
Meall nan Tarmachan, Mid-Perth	***C. micacea***	NN/592.408	26	8	0	600m	*Festuca ovina, Alchemilla alpina, Saxifraga azoides, Oxyria digyna, Parnassia palustris*
Gordale Scar, Mid-West Yorkshire	*C.p* subsp. *pyrenaica*	SD/914.639	12	5	10	300m	A sparse plant community, species not recorded.

Table 4.2: Upland *Cochlearia* sample sites, showing the altitude at which the collections were made and any recorded chromosome counts for that locality chromosome counts by Gill (1973, 1978). Populations confirmed as *C. micacea* (Dalby & Rich 1994) are shown in bold.

4.2.4 Chloroplast RFLP variation

Initial screening for polymorphisms was carried out using eight *Cochlearia* samples from a range of taxa and geographical regions: *C. officinalis* subsp. *scotica*: Uig5 (NB/050.329), Port GheirahaS2 (NB/355.499); *C. pyrenaica* subsp *alpina* Hart Fell Rig8 (NT 119 141), Cwmbrynog4 (SH/601.555)]; *C. micacea*: Ben Dothaidh6 NN/326 411; *C. atlantica*: Fort William7 (NN/087.764); *C. officinalis* s.s.: Sand bay6 (ST/322.630); *C. danica*: Llanwit Major8 (SS/957.674). Five chloroplast regions were amplified: psbC-trnS, $trnK_1$ -$trnK_2$, petD-petB, psbC-trnT, psbA-trnS. 8 different restriction enzymes was trialled with the five chloroplast regions: *Alu* I, *Hinf* I, *Mbo* I, *Rsa* I, *Taq* I, *Msp* I, *Hae* III, *Mse* I. Variation was found only in the trnK-K region using Rsa I and Mse I, which detected polymorphisms separating the same groups of samples, which was probably attributable to a single insertion/deletion.

The whole data set was screened for polymorphisms only in the $TrnK_1$ -$TrnK_2$ region using the enzyme Rsa1. The amplification reactions were performed in 25µl, with 1x taq buffer (Bioline: 16mM ($(NH_4)_2SO_4$), 67mMn Tris-HCl (pH8.8), 0.01% Tween-20), 2mM $MgCl_2$ (Bioline), 100µM dNTPs (Sigma), 200µM of forward and reverse primer (MWG Biotech), 2µg bovine serum albumin (Promega) and 1unit of taq DNA polymerase (Bioline) under the following conditions: 94ºC for 4 mins, 30-35 cycles of 92ºC 45 seconds, 56ºC for 45 seconds, 72ºC for 2 minutes, followed by a final extension at 72ºC for 10mins. The PCR products were checked for successful amplification of the target region in agarose gels stained with SYBRSAFE™.

The PCR products were digested with Rsa1 (New England Biolabs). Digestions were carried out in 20µl reactions at 37ºC for 3 hours. The digestion mix was as follows: (50mM NaCl, 10nM Tris-HCl, 10mM $MgCl_2$, 1mM DTT (pH 7.9)), 5 U restriction enzyme and 5µl of PCR product.

5µl of the digested PCR products were separated by electrophoresis through 8% polyacrylamide gels (Sambrook *et al.* 1989). A 1kb ladder was included as a size reference. Gels were run in 1xTBE at 20V/cm for 2.5 hours in a vertical gel apparatus**.** The gels were then visualised under ultraviolet light after staining with a solution of 10% SYBRSAFE™ in TBE buffer.

4.2.5 AFLP marker generation

All populations (see Table 2) were scored for variation at AFLP marker loci. The AFLP analysis was carried out as in Chapter 2.

4.2.6 Data analysis

4.2.6.1 Analysis of morphological data

The leaf measurements were made as described in Chapter 3. Leaves from all populations were measured except for Meall nan Gabhar and Meall nan Tarmachan. The leaf data was analysed as follows: Boxplots were created in the software package MINITAB® 14 (Minitab Inc.) to show the variation within and between populations and taxa for each morphological character. The Little Kilrannoch population was not given a species classification in the boxplots because of its unusual growing conditions and atypical morphology. Principal components analysis was used on the morphological data. Nested general linear model (GLM) analysis of variance (ANOVA) was used to calculate the variance in morphological characters, among populations and among regions and taxa. A PCA plot was used to test whether the characters combined discriminated between the putative species.

4.2.6.2 Analysis of AFLP

The AFLP data was handled and analysed as in Chapter 3. The following groupings were used for PCO analysis: population, taxon and *C. micacea* vs. non-*C. micacea* populations. AMOVA analysis was used to detect variation in AFLP fragments between the following groupings: among populations, among taxa, between *C. micacea* and non-*C. micacea* groupings. A Mantel test was used to test for a correlation between genetic and geographical distance.

4.2.6.3 Detailed analysis of AFLP and morphological data from the Ben Lawers population

The length and width of the seed pods on each plant collected was measured. The mean average pod length and width for each plant was then converted into a length: width ratio. The pod length: width ratio was used to quantify the ‘narrowness’ of pods. Jaccard’s similarity coefficient was calculated from AFLP data to serve as an input value for PCO analysis, and then the results were displayed as a scatterplot. The score for each individual was taken from axis one to summarise individual similarity and variation in the genetic

data. This score was correlated (Pearson Correlation in MINITAB) with the pod length: width ratio data, to compare genetic similarity with pod shape similarity

4.3 Results

4.3.1 Cultivated *Cochlearia* from study populations

Specimens of plants sampled from upland populations grown in standard conditions in the glasshouse are shown in Figures 4.2-4.9.

Plants from five of the upland populations were very similar morphologically when grown in standard conditions. The populations of origin were: Beinn Ghlass (Ben Lawers NNR-Figure 4.2), Beinn Heasgarnich (Figure 4.3), Cairnwell (Figure 4.4), Meall nan Tarmachan (Figure 4.6) and Little Kilrannoch (Figure 4.7). The Ben Lawers populations were identified as *C. micacea*, as were the Beinn Heasgarnich and Meall nan Tarmachan populations. The Cairnwell and Little Kilrannoch populations were identified as *C. pyrenaica* subsp. *alpina* based on field morphology; however they were more similar to *C. micacea* populations when plants of both taxa were grown under standard conditions. The Little Kilrannoch population in particular had a very different morphology when grown in the glasshouse compared with the wild population (Figure 4.8). The Lochnagar specimen (Figure 4.9) was morphologically distinctive from all the other populations, with large angular leaves and long stems. In the wild population the Lochnagar plants were smaller and more *C. micacea*-like. The plants from Gordale Scar (Figure 4.5) had larger, more deeply cordate leaves than the *C. micacea* populations. The difference in morphology was expected here because this population was a putative diploid population.

Figure 4.2: Plant from Beinn Ghlass (Ben Lawers NNR) putative taxon: *C. micacea*

Figure 4.3: Plant from Beinn Heasgarnich, putative taxon: *C. micacea*

Figure 4.4: Plant from Cairnwell, putative taxon: *C. pyrenaica* subsp. *alpina*

Figure 4.5: Plant from Gordale Scar, putative taxon: *C. pyrenaica* subsp. *pyrenaica*

Figure 4.6: Plant from Meall nan Tarmachan, putative taxon: *C. micacea*.

Figure 4.7: Plants from Little Kilrannoch, putative taxon: *C. pyrenaica subsp. alpina*

Figure 4.8: Plants growing wild at Little Kilrannoch, putative taxon: *C. pyrenaica* subsp *alpina*.

Figure 4.9: Plants from Lochnagar, putative taxon: *C. pyrenaica* subsp. *alpina*.

4.3.2 Results of leaf measurements.

All of the morphological characters showed considerable variation within each population and taxon and it was difficult to identify discontinuous morphological differences between populations. The box plots showed there was a trend for *C. pyrenaica* subsp. *alpina* and *C. pyrenaica* subsp. *pyrenaica* plants to have longer leaves than *C. micacea* plants (Figure 4.10). The longest and widest leaves were found in the Lochnagar populations (Table 4.3 and Figure 4.10). The shortest and narrowest were found in the Little Kilrannoch population (Table 4.3 and Figures 4.10 and 4.11). Leaf length: width (Figure 4.12) varied between taxa. Leaf-base angle (Figure 4.13) did not seem to vary according to taxonomic groupings, although the leaf-base angle of the Little Kilrannoch plants was much smaller than in the other populations.

The GLM ANOVA analysis of leaf morphology (Table 4.5) showed there was a significant difference between leaf length leaf, leaf width, and leaf base angle between the sampled populations. Only leaf length: width was significantly different among taxa.

The plotted PCA scores (Figure 4.14) for the morphological variables combined showed that when used together the characters do not clearly distinguish between the taxa; however, there was a weak tendency for *C. micacea* samples to group to the left. The AMOVA (Table 4.6) shows that there was no significant variation in PCA scores derived from morphological variables between the three taxonomic groups, although there was significant variation between the populations.

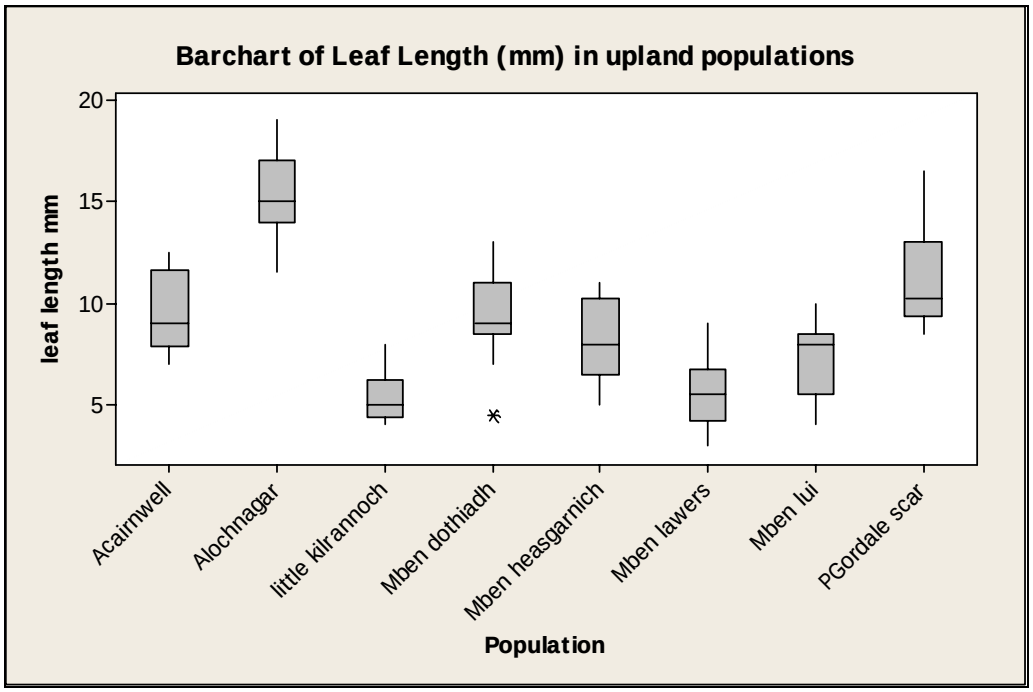

Figure 4.10: Box-plots showing the variation in leaf length among upland *Cochlearia* taxa and populations. The taxon is shown by the letter prefix on the population name: M = *C. micacea*, A = *C. pyrenaica* subsp. *alpina*, P = *C. pyrenaica* subsp. *pyrenaica*.
* = outlier

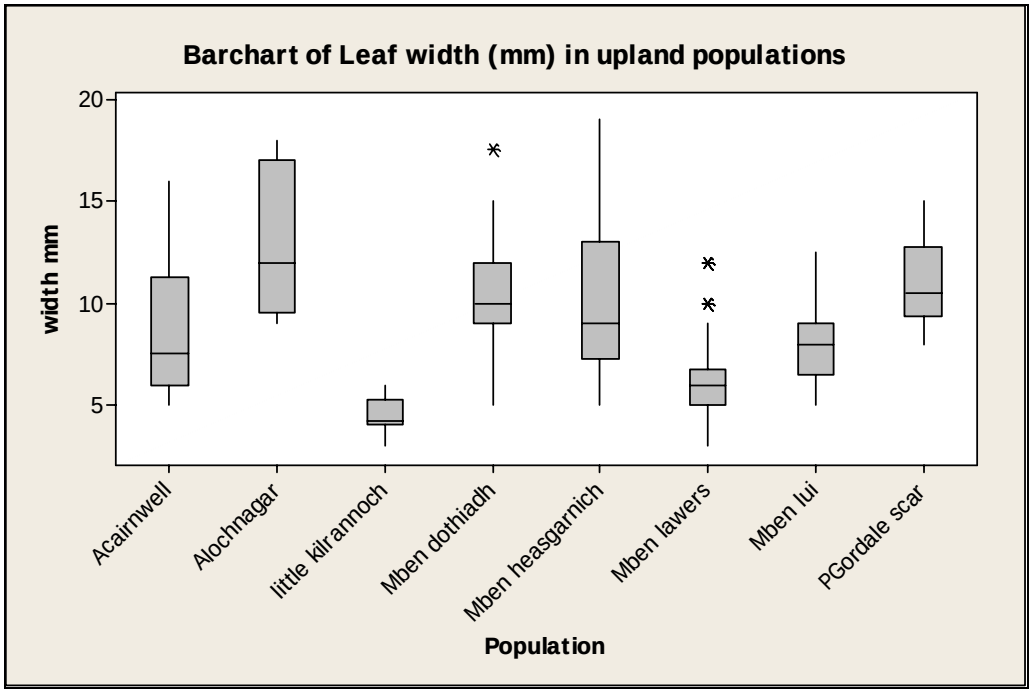

Figure 4.11: Box-plots showing the variation in leaf width among upland *Cochlearia* taxa and populations. The taxon is shown by the letter prefix on the population name: M = *C. micacea*, A = *C. pyrenaica* subsp. *alpina*, P = *C. pyrenaica* subsp. *pyrenaica*.
* = outlier

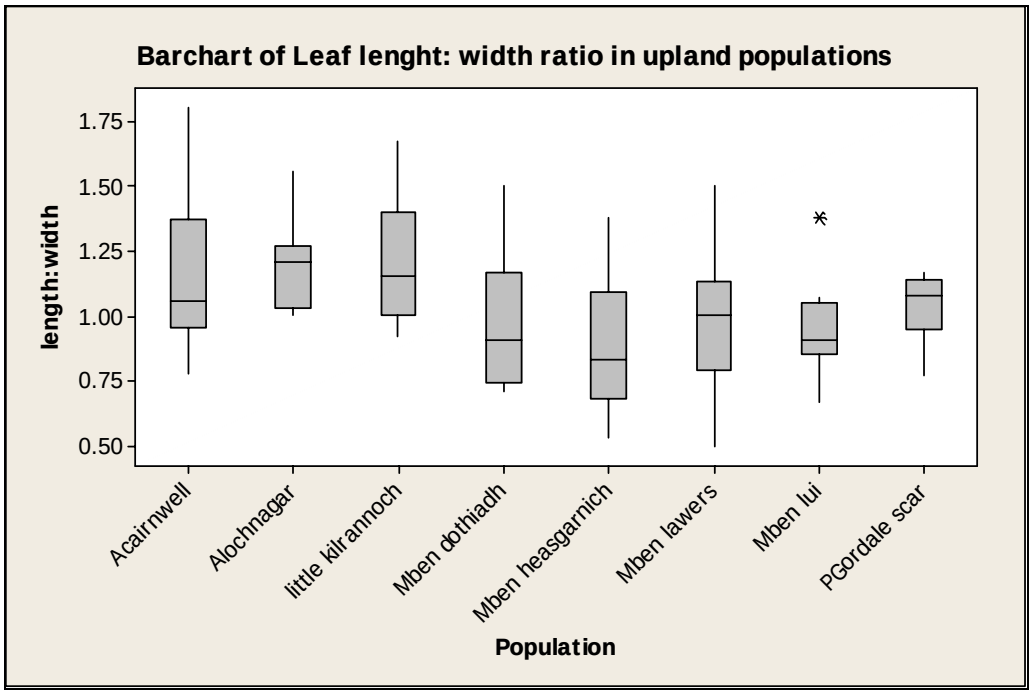

Figure 4.12: Box-plots showing the variation in leaf length:width ratio among upland *Cochlearia* taxa and populations. The taxon is shown by the letter prefix on the population name: M = *C. micacea*, A = *C. pyrenaica* subsp. *alpina*, P = *C. pyrenaica* subsp. *pyrenaica*. * = outlier

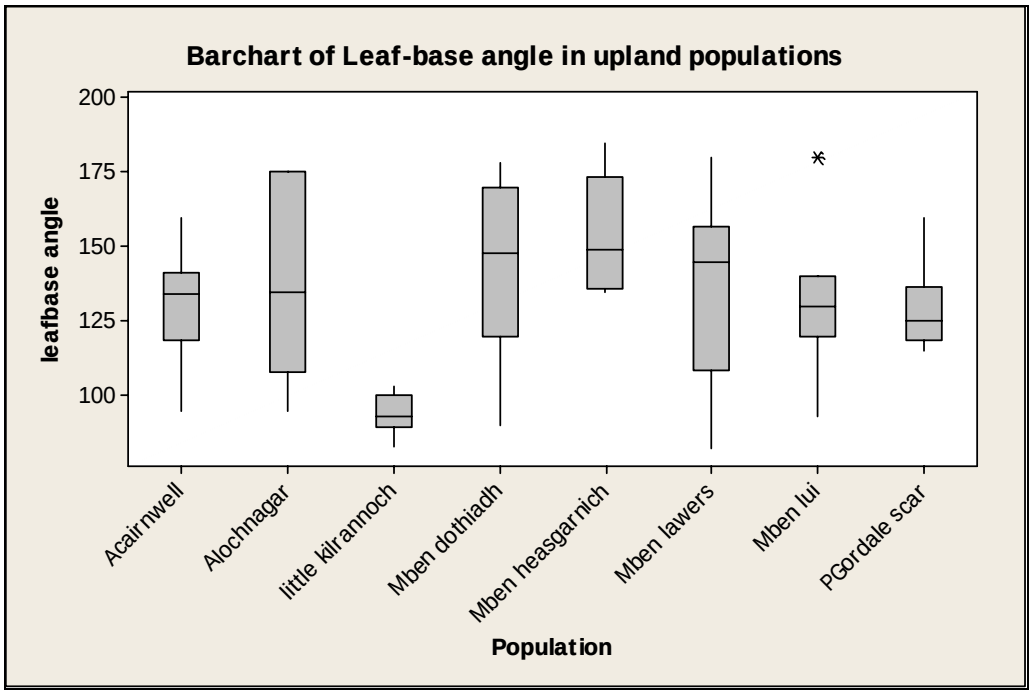

Figure 4.13: Box-plots showing the variation in leaf-base angle among upland *Cochlearia* taxa and populations. The taxon is shown by the letter prefix on the population name: M = *C. micacea*, A = *C. pyrenaica* subsp. *alpina*, P = *C. pyrenaica* subsp. *pyrenaica*. * = outlier

Population	Leaf length (mm)	Leaf width (mm)	Leaf length:width	Leafbase angle
Ben Lawers	5.7 (±1.6)	10.1 (±3.3)	1.0 (±0.3)	135.7 (±30.2)
Ben Lui	7.4 (±1.8)	7.9 (±1.9)	0.9 (±0.2)	129.4 (±20.3)
Cairnwell	9.5 (±2.0)	8.7 (±3.4)	1.2 (±0.3)	130.3 (±17.8)
Ben Heasgarnich	8.2 (±2.1)	10.2 (±4.8)	0.9 (±0.30)	154.1 (±20.6)
Lochnagar	15.1 (±2.4)	13.1 (±3.6)	1.2 (±0.2)	138.3 (±30.8)
Ben an Dothaidh	9.3 (±2.0)	10.1 (±3.3)	1.0 (±0.2)	142.3 (±28.0)
Little Kilrannoch	5.4 (±1.3)	4.6 (±0.7)	1.2 (±0.3)	94.1 (±6.20)
Gordale Scar	11.3 (±2.8)	11.1(±2.4)	1.0 (±0.2)	128.8 (±13.6)

Table 4.3: Table of mean average and standard deviation of leaf measurements for each population of upland *Cochlearia*.

Taxon	Leaf length (mm)	Leaf width (mm)	Leaf length:width	Leafbase angle
C. micacea	7.1 (± 2.3)	7.9 (±3.2)	1.0 (±0.2)	137.5 (±27.3)
C. pyrenaica **subsp*. alpina***	9.4 (±4.3)	8.3 (±4.6)	1.2 (±0.3)	119.0 (±27.0)
C. pyrenaica **subsp. *pyrenaica***	11.3 (± 2.8)	11.1 (±2.7)	1.0 (±0.1)	128.8 (±13.6)

Table 4.4: The mean average and standard deviation for leaf measurements among three upland *Cochlearia* taxa.

Source of variation	Degrees of freedom	Leaf length (mm)	Leaf width (mm)	Leaf length:width	Leafbase angle
		MS	MS	MS	MS
Taxon	3	166.91	107.67	0.370***	5825.30
Population	5	53.69***	53.58***	0.018	2596.60***
Error	93	3.76	7.59	0.052	487.70
Total	101				

Table 4.5: General linear model ANOVA results for morphological variables for upland *Cochlearia* taxa and populations. (* = P value < 0.05, ** = P value < 0.01, *** = P value 0.001)

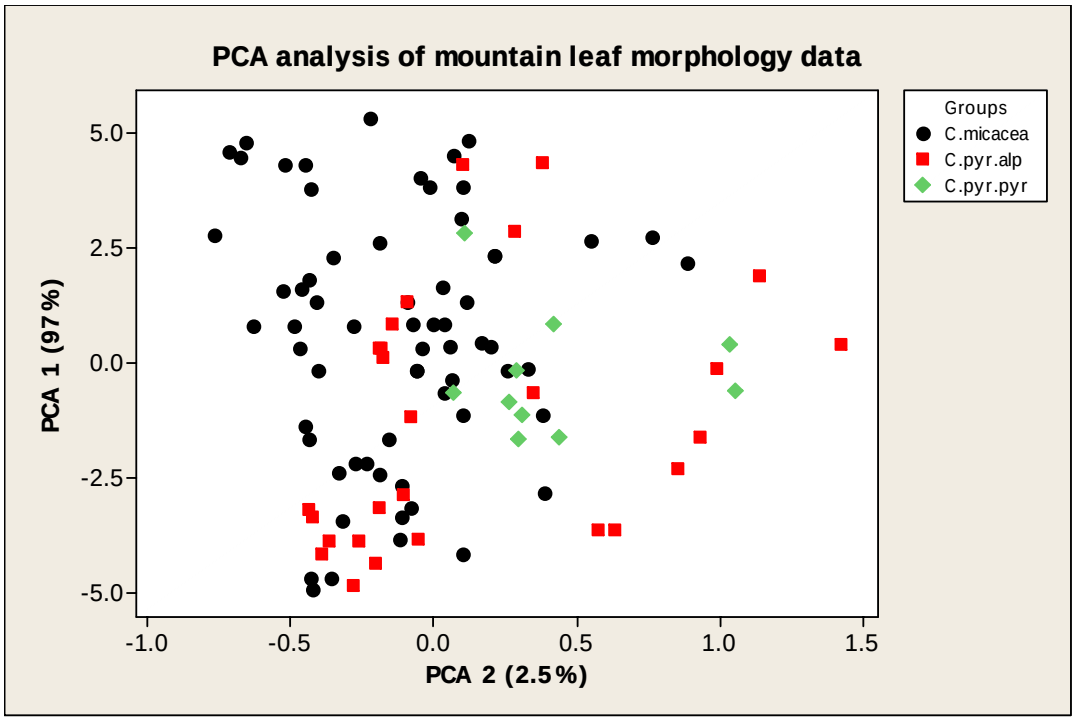

Figure 4.14: A scatter plot showing all leaf morphological variables for three sampled upland *Cochlearia* taxa combined and converted to PCA scores. Axis 1 was plotted against Axis 2. The colours represent individuals from the three taxa.

Source of variation	DF	MS
Taxon	2	32.40
Between populations within taxa	5	26.58***
Error	94	5.79
Total	101	

Table 4.6: ANOVA of first principal component score based on field characters for three upland *Cochlearia* taxa. (* = P value < 0.05, ** = P value < 0.01, *** = P value 0.001)

4.3.3 Results for cp PCR-RFLP

The initial screening showed the variation in the chloroplast region among the upland populations was very low. Variation was found in only one region ($TrnK_1$-$TrnK_2$) out of 5 regions. Three haplotypes were detected. All but seven samples out of ninety-five total were of the same haplotype. Two (out of 23) samples from Ben Lawers (Figure 3.15) and one (out of 10) sampled from Ben an Dothaidh (Figure 3.16) share the rare haplotype B. Three (out of 8) Gordale scar samples had the rare haplotype C (Figure 3.19). The other populations screened were exclusively haplotype A (Figures 4.17 and 4.18).

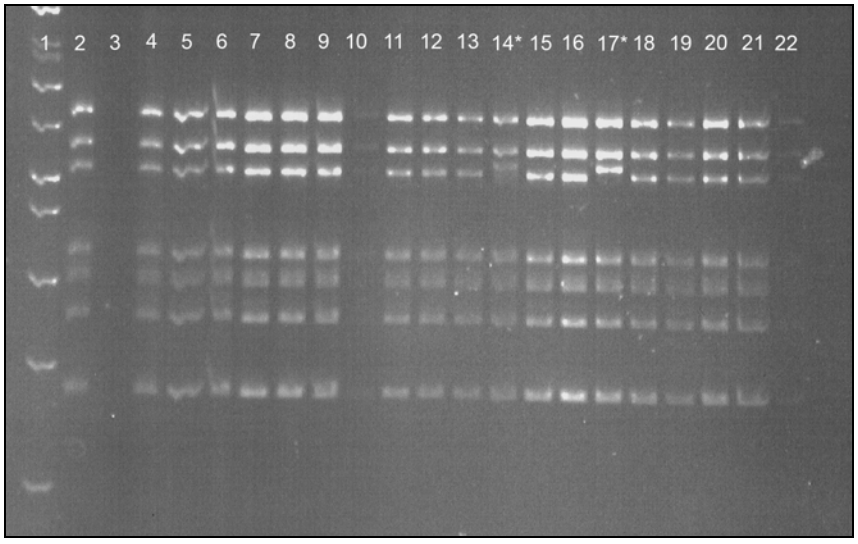

Figure 4.15: 1=Bioline™ 1kb ladder, 2-13 Ben Lawers population *BL1-12*, 13-22, Ben Lawers population *BLW2-8, 10, 9, 11*. Haplotype 'B' in lanes 14 & 17 (BLW 3, 6) lanes marked with an asterisk.

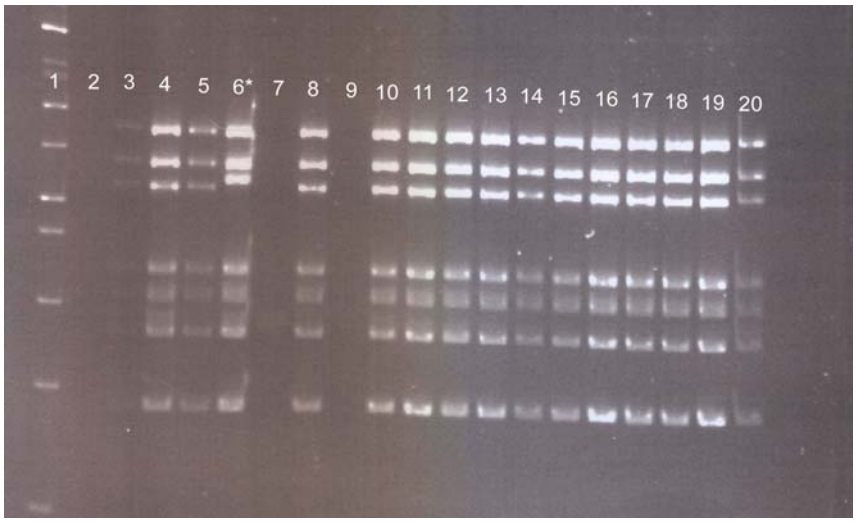

Figure 4.16: 1 = ladder, 2, 3: Ben Lawers population 2: *BLW 12, 13*, 4-11: Ben an Dothaidh *BD3- BD10*, 12-19: Cairnwell *CRW 1-8*, 20: Beinn Heasgarnich *BHE 1*. Haplotype 'B' in lane 6 (BD5) lanes marked with an asterisk

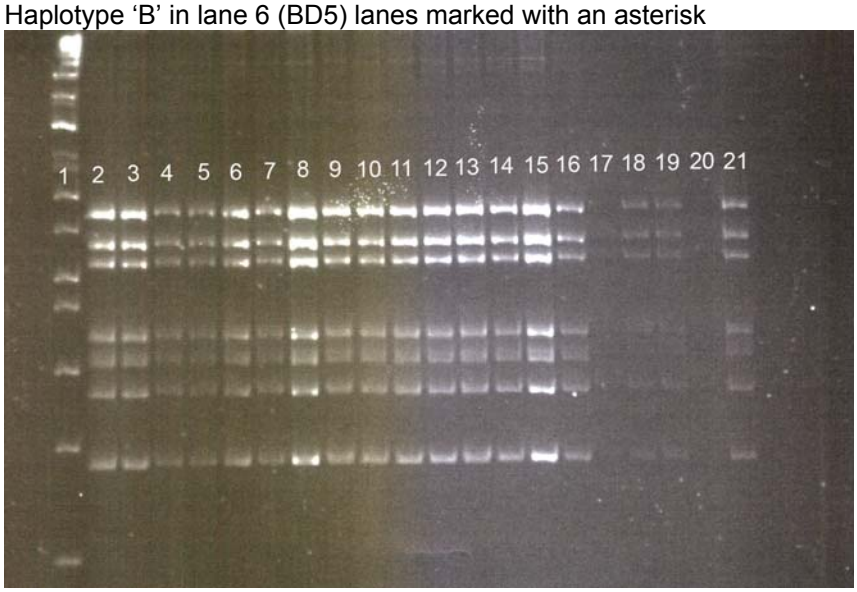

Figure 4.17: 1 = ladder, 2-8: Beinn Heasgarnich *BHE 2-8*, 9-16: Lochnagar *LNG 1-8*, 17-21: Ben Lui *BLU1-5*. No polymorphism found.

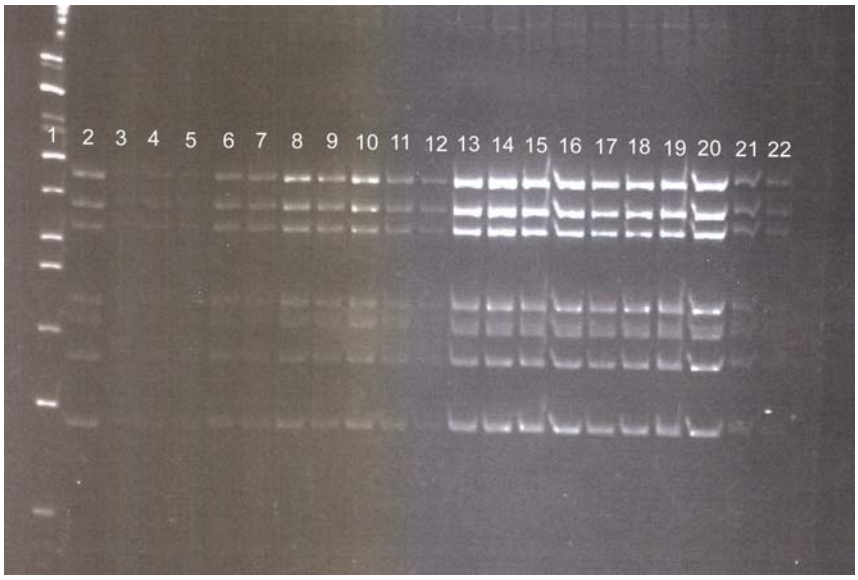

Figure 4.18: Chloroplast PCR-RFLP gel for the following samples - 1 = ladder, 2-4: Ben Lui *BLU6-8*, 5-12 Meall nan Gabhar *MNG 5-12*, 13-20 Little Kilrannoch *LKR1-20*, 21-22 21, 22: Meall nan Tarmachan *MNT1-2*. No polymorphism found.

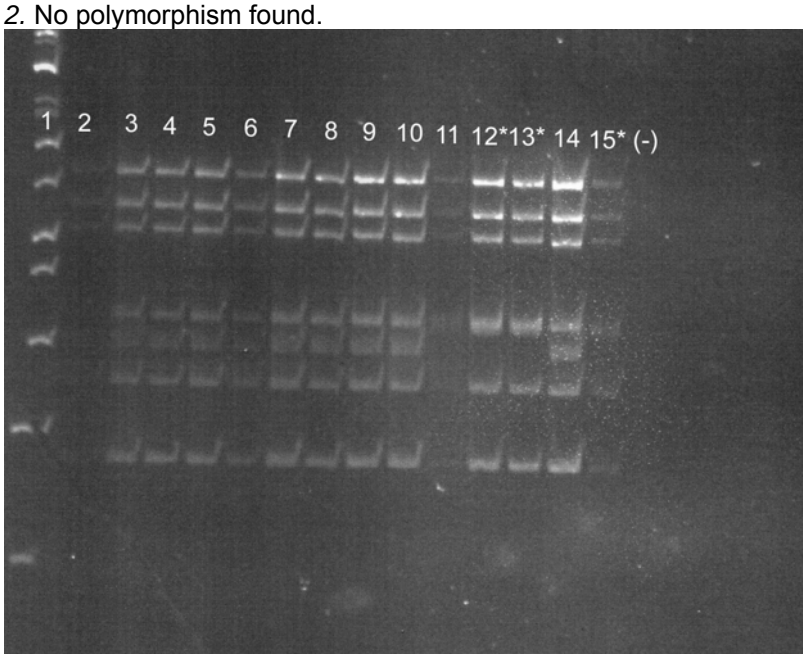

Figure 4.19: 1 = ladder, 2-8 Meall nan Tarmachan, *MNT 3-8*, 8-15 Gordale Scar *GS1-8*, 16 Negative Control. Polymorphism 'C' in lanes 12, 13 (GS 5, 6) lanes marked with an asterisk.

4.3.4 Results from AFLP analysis of fragment frequency diagnostic and private fragments

The AFLP analysis produced 255 polymorphic scorable fragments. The highest average fragment number (65.6) among samples was derived from the Beinn Heasgarnich population (Table 4.7). The lowest mean average fragment number was much lower (21.8) and was from the samples of the Little Kilrannoch population (Table 4.7). All populations had one or more private fragments, although most were at low frequency (Table 4.7). There were two private fragments (one diagnostic) in the Beinn Heasgarnich population that were present at high frequency. There was one private fragment in the Meall nan Tarmachan population that was present at high frequency. Gordale Scar, a diploid population, produced a similar number of fragments to the tetraploid populations. Ben Lawers1 had the greatest number of private fragments, although none of these fragments were found at high frequency (>50% samples). More than half were present in more than one sample. Lochnagar had the lowest number of private fragments, with only one.

The average fragment number for the three taxa is shown in Table 4.8, along with the distribution of private fragments by taxon. *C. pyrenaica* subsp. *alpina* samples produced the greatest average number of fragments (52.4). *C. micacea* samples produced the smallest average number of fragments (47.3), but by far the greatest number of private fragments. The general linear model (GLM) ANOVA (Table 4.9) showed there was a significant difference in fragment number among populations, but not between taxa.

Population	Mean number of fragments	Number of fragments polymorphic above 5% level	Number private fragments	Number diagnostic fragments	Private fragments in >50% of population	Private fragments present in one sample (singletons)	% Singleton private fragments
Benlawers1	42.2	37.3	16	0	0	11	68.75
Benlawers2	45.2	51.0	8	0	0	8	100
Ben an Dothaidh	53.6	42.4	6	0	0	6	100
Ben Heasgarnich	65.6	45.5	7	1	1	4	57
Meall Nan Tarmachan	55.4	45.9	2	0	1	2	100
Ben Lui	37.8	43.5	3	0	0	3	100
Cairnwell	49.6	37.3		0	0		
Gordale Scar	52.4	39.6	4	0	0	4	100
Little Kilrannoch	21.8	29.8	6	0	0	6	100
Meall nan Gabhar	57.3	44.7	4	0	0	1	25
Lochnagar	55.4	46.7	1	0	0	1	100

Table 4.7: Mean AFLP fragment number, with distribution of private and diagnostic fragments among populations upland *Cochlearia* from three taxa.

Taxon	Mean number of fragments	Private fragments	Present in >50%	Number of fragments in only one sample
C. micacea	47.9	83	0	43
C. pyrenaica* subsp. *pyrenaica	49.3	5	1	3
C. pyrenaica* subsp. *alpina	52.4	11	0	5

Table 4.8: Mean fragment number, with distribution of private and diagnostic AFLP fragments among three upland *Cochlearia* taxa

Source	DF	MS
Taxa	2	46.90
Between population within taxa.	8	1207.73***
Error	78	81.33
Total	88	

Table 4.9: GLM nested ANOVA showing variation in AFLP fragment number between upland taxa and populations. (* = P value < 0.05, ** = P value < 0.01, *** = P value 0.001).

4.3.5 AFLP variation

The first two axes of the PCO plots (Figures 4.20 and 4.21) explain 17.8% of the variation. There did not appear to be clear clustering of populations or individuals by geography (Figure 4.20). There was a tendency for putative *C. pyrenaica* subsp. *alpina* populations to group in the bottom left of the plot (Fig. 4.20). The population from Gordale scar (*C. pyrenaica* subsp. *pyrenaica*) was nested within the variation of the other two taxa (Figure 4.20). The population that produced the greatest number of fragments (Beinn Heasgarnich) and the population that produced the smallest number of fragments (Little Kilrannoch) are furthest apart in the PCO plot (Figure 4.20). *Cochlearia micacea* and non-*C. micacea* populations tended to group separately (Figure 4.21), but there was a big overlap between the two groups. The AMOVA (Table 4.10) between the two *C. micacea* and non-*C. micacea* types was not significant, giving little support for separate taxonomic grouping of *C. micacea* and non-*C. micacea* populations. However, there was significant variation among populations within named taxa.

The patterns of differentiation as inferred from Φst values (Table 4.11) are complex and do not fit taxonomic or geographical predictions. The average Φst for populations across all loci was Φst = 0.179 ($P < 0.0001$). The Meall nan Ghabar population had the highest average pairwise Φst = 0.26. There were some very low Φst values that were not significantly different from each other between relatively geographically close populations. All of the population pairs with low pairwise Φst values are geographically close together – Ben Lawers1 & Ben Lawers2 and Ben Lui in particular. Overall there did not appear to be a relationship between Φst values and geographical distance. This was supported by the results of the Mantel test, which showed no significant relationship ($r^2 = 0.004$) between genetic and geographical distance. High pairwise Φst values were derived for the little Kilrannoch population compared with the Cairnwell population (the closest geographically), Beinn Heasgarnich, Lochnagar and Meall nan Gabhar, but very low differentiation from Ben Lui. Ben an Dothaidh had a very low pairwise Φst value with Ben Lawers2, but a higher one with Ben Lawers1, with whom it shared a chloroplast haplotype (Figures 4.14 and 4.15).

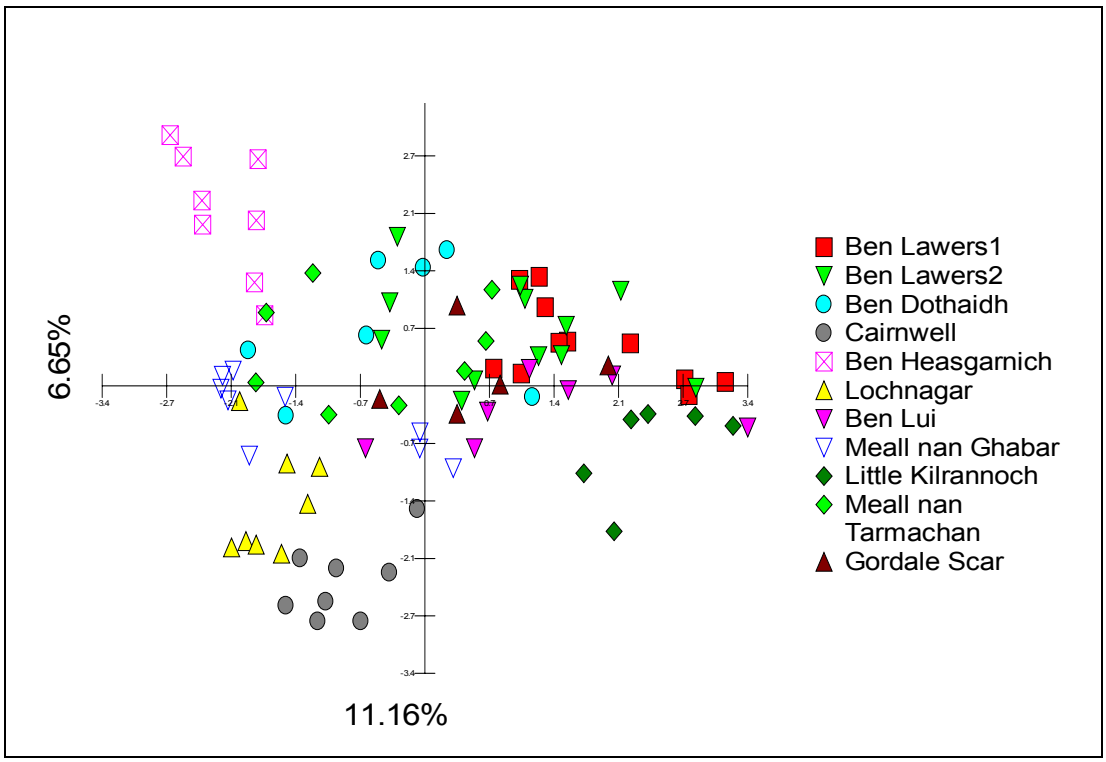

Figure 4.20: PCO analysis plot showing the phenetic relationships between eleven upland *Cochlearia* populations of three taxa: *C. micacea*, *C. pyrenaica* subsp. *pyrenaica*, *C. pyrenaica* subsp. *alpina* based on AFLP variation converted to Jaccard's similarity coefficients.

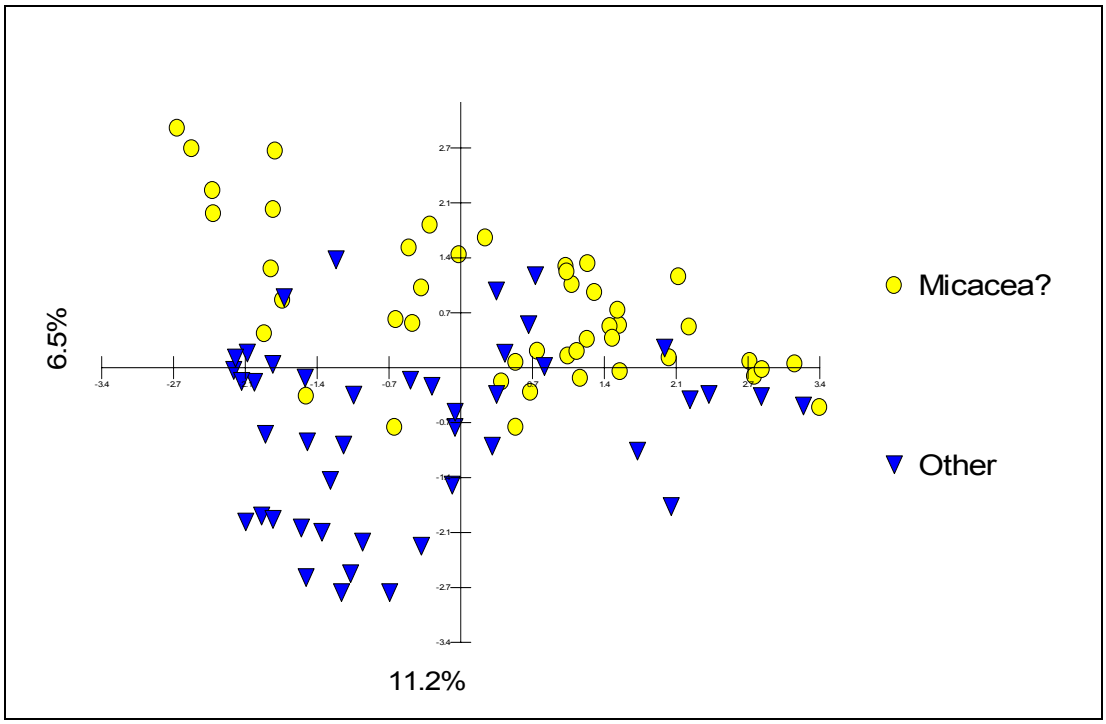

Figure 4.21: PCO plot showing phenetic relationships between eleven upland *Cochlearia* populations of three taxa. *C. micacea* populations are compared with the other upland populations AFLP variation converted to Jaccard's similarity co-efficients.

AMOVA MICACEA V OTHERS	Degrees of freedom	Sum of squares	Variance components	% of variation	P values
Among groups	1	70.04	0.34	1.39	0.24
Among pops within groups	9	478.98	4.13	17.06	<0.0001
Within populations	78	1559.98	19.75	82.06	
Total	89	2108.99	24.21		

Table 4.10: Table showing AMOVA results for AFLP variation between *C. micacea* populations compared with non-*C. micacea* populations.

	Benlawers1**M**	Benlawers2**M**	Bendoth**M**	Cairnwell**A**	BenHeas**M**	Lochnagar**A**	Ben Lui**M**	MealInGhab**A**	Littlekil**A**	MealInTar**M**	Gorscar**P**
Benlawer1**M**	0										
Bbenlawer2**M**	0.026ns	0									
Bendoth**M**	0.107	-0.00013ns	0								
Cairnwell**A**	0.255	0.205	0.181	0							
BenHeas**M**	0.315	0.270	0.215	0.298	0						
Lochnagar**A**	0.255	0.194	0.123	0.149	0.213	0					
Ben Lui**M**	0.105	0.107	0.096	0.169	0.251	0.176	0				
MealnGhab**A**	0.253	0.232	0.204	0.268	0.252	0.179	0.143	0			
Littlekil**A**	0.148	0.133	0.185	0.261	0.338	0.269	0.00111ns	0.244	0		
MealnTar**M**	0.146	0.109	0.077	0.224	0.223	0.166	0.083	0.132	0.150	0	
Gorscar**P**	0.130	0.150	0.128	0.231	0.272	0.227	0.113	0.215	0.149	0.140	0
Mean Pop. Average	0.174	0.133	0.141	0.194	0.265	0.195	0.121	0.212	0.188	0.145	0.169

Table 4.11: Table of Pairwise Φst's between the ten sampled upland populations. **M** = *C. micacea*, **A** = *C. pyrenaica* subsp. *alpina*, **P** = *C. pyrenaica* subsp. p*yrenaica*. ns = non-significant at the P = 0.05 level, all unmarked values are significant to < P = 0.05

4.3.6 Analysis of two populations at Ben Lawers

4.3.6.1 Analysis of pod shape distribution

Each population contained a mixed array of pod shapes, none of which had conspicuous veins. Pods were of mixed shape and size in both populations and there was no evidence of two discrete classes of pod shape in the histogram of pod length ratio (Fig 4.22). There were no significant differences between pods between the two populations for any characters (Table 4.13); the mean average pod measurements for each population are also shown in Table 4.12. There was no correlation between pod length: width ratio (as a proxy for narrowness) and genetic similarity (converted to a PCO score): axis 1: R = -0.147, P = 0.505 (Pearson's correlation).

4.3.6.2 AFLP variation at Ben Lawers

Both populations had a high number of fragments that were not present in the other population (Table 4.14). In population 1, 49.57 of the 97 shared fragments were high frequency - present in more than 25% of all upland populations and 34/57 are present in 50% or more of all the samples.

Despite the number of private fragments each population had, the differentiation in variation between the two populations was Φst = 0.026 (or accounting for 2.6% of the variation - Tables 4.10 and 4.12). The two populations at Ben Lawers (see Figure 4.22) showed some separation in genetic similarity when other upland populations were excluded. However the variance between the two populations was non-significant (Table 4.15).

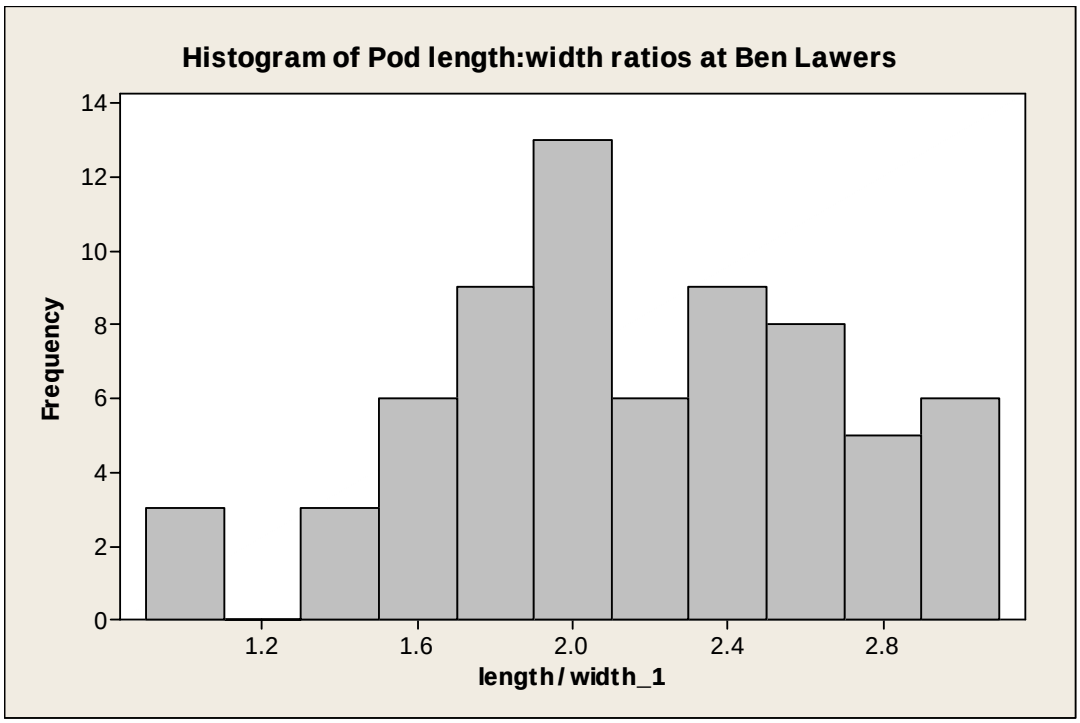

Figure 4.22: Histogram showing the frequency distribution of pod length: width ratios among both Ben Lawers populations combined.

Measurement	Population 1 (S.D)	Population 2 (S.D)
Mean average pod width (mm)	2.5 (±0.5)	2.5 (±0.4)
Mean average pod length (mm)	5.0 (±0.9)	5.4 (±1.0)
Mean average pod length: width (mm)	2.1 (±0.5)	2.2 (±0.5)

Table 4.12: Mean average and standard deviation of pod measurements for the two putative *C. micacea* Ben Lawers populations.

Source of Variation		Pod width	Pod length	Pod length:width
	DF	MS	MS	MS
Population	1	0.06	2.68	0.15
Error	66	0.23	0.93	0.27
Total	67			

Table 4.13: ANOVAs of pod dimension between the two putative *C. micacea* populations at Ben Lawers. None had P-values less than P=0.05.

Ben Lawers population	Private fragments	Shared fragments
Population 1	49	97*
Population 2	35	

Table 4.14: The number of private and shared AFLP fragments between the two putative *C. micacea* populations at Ben Lawers.

Source of variation	D.F	Sum of squares	Variance	% of variation	P-values
Among populations	1	27.46	0.5650	2.62	0.12
Within populations	21	440.46	20.9740	97.38	
Total	22	467.91	21.5390		

Table 4.15: AMOVA of AFLP variation between two populations of putative *C. micacea* at Ben Lawers.

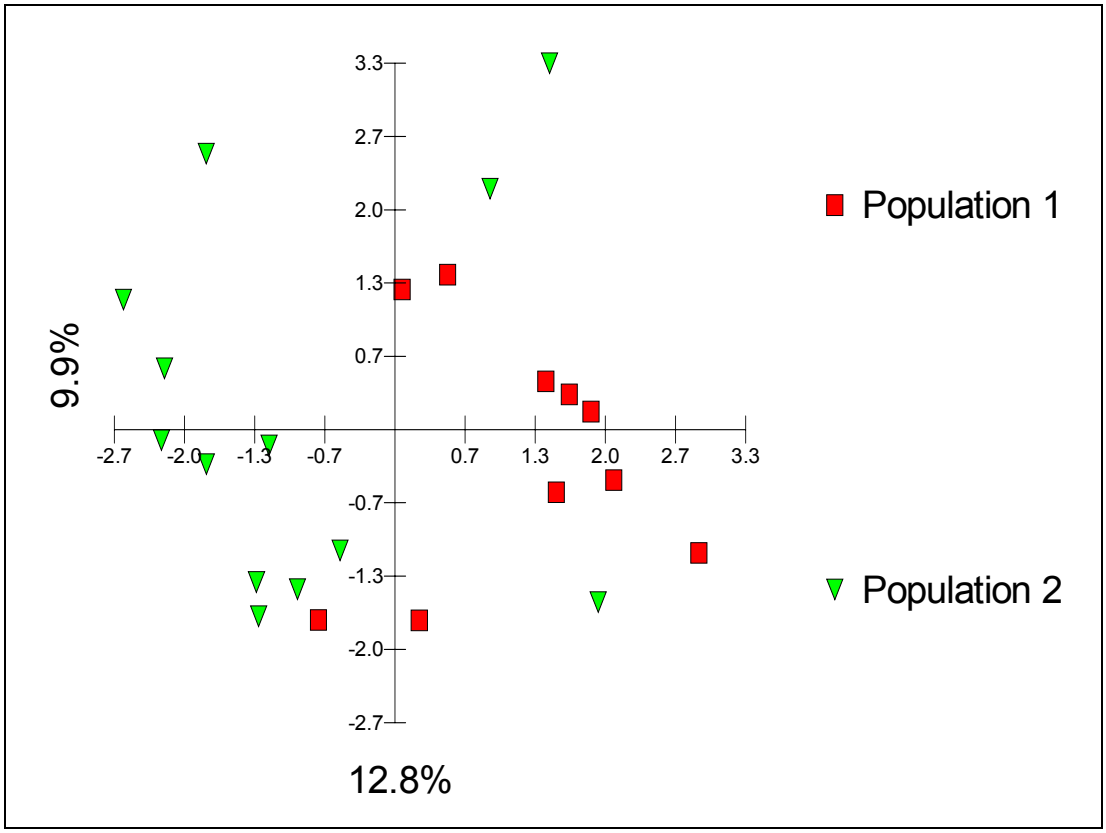

Figure 4.23: A scatter plot showing the phenetic relationships between the two populations of putative *C. micacea* at Ben Lawers based on PCO analysis of Jaccard's similarities of AFLP data.

4.4 Discussion

4.4.1 Morphological variation.

Morphological plasticity may account for some of the variation in wild populations. The cultivated plants from Lochnagar are morphologically distinctive compared with the other populations in cultivation. Cairnwell and Little Kilrannoch appear to be morphologically similar to *C. micacea* populations under standard conditions, despite being identified as *C. pyrenaica* subsp. *alpina* on the basis of morphological characters in the field.

Some plants from Little Kilrannoch and Ben Lawers were very uniformly small in the field; however in the nursery they grew much more vigorously, suggesting that environmental factors were limiting the size of wild populations. Both of the populations that were small in the field came from particularly challenging habitats. The plants from the summit population of Ben Lawers spend months under the snow and are subject to harsh, exposed conditions year-round. The plants from the summit of Little Kilrannoch grow on free-draining serpentine gravel, which has a high heavy metal content. The populations from a sheltered gully on Ben an Dothaidh did not show size differences between the wild and cultivated populations.

The morphological analysis of leaf shape in wild populations shows that there is considerable morphological variation between populations of the three upland *Cochlearia* taxa. The variation present is almost continuous, although the leaf length: width ratio did vary between taxa. However, if all the morphological characters are combined they do not effectively separate taxa. The analysis of leaf characters did not yield suites of characters for taxonomic groups. A more detailed morphometric study with a greater number of characters and representatives from each taxon may have illustrated the differences between taxa more convincingly.

4.4.2 Chloroplast RFLP variation

The same chloroplast haplotype was encountered in almost all the samples of *C. pyrenaica* and *C. micacea*. This is consistent with the work of Koch *et al.* (1996) who took samples over a wide geographical range across Europe and found only 4 restriction site mutations, characterising 6 haplotypes. It is possible that more extensive initial screening would have revealed a larger number of rare haplotypes, however due to the very low level of variation,

it is unlikely that phylogenetically or taxonomically informative chloroplast variation would have been found among the British *Cochlearia* samples in general.

The presence of the rare chloroplast mutation in the summit population at Ben Lawers and not in the second flush population is interesting. However, the mutation is only present in low frequency in the summit population, so lack of seed flow cannot be inferred. Additionally, the samples only represent a small fraction of each population, so we cannot say the mutation is definitely absent from the flush population. The same mutation was also present in the Ben an Dothaidh site 30km away. The most likely explanation for this is that these populations came from the same source. The source population may have been at a lower altitude, which was then separated and sub-sampled as the plants moved to a higher altitude during post glacial warming. There is no way of knowing the location of the source population from the information currently available.

4.4.3 Genetic variation.

The most striking difference in fragment number was found in samples from Little Kilrannoch which had a much smaller average number of fragments compared with the other populations (discussed in more detail in section 4.4.4). Studies using the AFLP technique with *Cardamine* (Lihova *et al.* 2003) and *Euphrasia* (French 2003) have detected differences in fragment number between plants of different ploidy levels. If chromosome number was an important factor in AFLP fragment numbers in *Cochlearia*, a reduced number of fragments would be expected for the diploid Gordale Scar population, but this does not occur. There were no differences in fragment number that could be attributed to chromosome number differences among the upland *Cochlearia*. This may be because they are allopolyploids rather than autopolyploids, which produces more copies of the same fragment, rather than more fragments.

There was no clear trend towards the grouping of AFLP variation by the three taxa or by *C. micacea* populations compared with the two subspecies of *C. pyrenaica.* The polarisation of Beinn Heasgarnich and Little Kilrannoch (with the greatest and the fewest number of fragments respectively) on the PCO plot suggested that the strongest factor in phenetic grouping was fragment number. Φst values were normal for an out-crossing plant (pairwise population average Φst = 0.12-0.26), suggesting some gene flow between populations. The average for insect pollinated out-crossing plants is ~0.20 (from allozyme data - Hamrick and Godt 1990). Many of the mountain populations are relatively isolated, so this could be

attributed to ancestral similarity between populations. The Φst values were much lower than those found in another AFLP study on upland *Cochlearia* in Eastern Europe (2002). In the Eastern European study Fst = 0.46-0.55, however the samples were collected over a much larger geographical area and the results were calculated from isozyme results and so are not strictly comparable with the results presented in this chapter.

The overall Φst value for the upland populations (Φst = 0.179) was slightly lower than the differentiation seen between coastal populations (Φst = 0.195). The pairwise geographical distance between upland populations was 101km among the upland populations and the average distance between coastal sites 295km. The coastal populations had similar pairwise Φst values, whilst being on average around 3 times further away from each other, suggesting there may be higher gene flow, or ancestral similarity between coastal populations. The difference in population subdivision is not as clear as might be expected between mountain and coast considering the much greater barriers to gene flow that exist in the mountains. Higher gene flow around the coast would make biological sense because seeds can move more easily between coastal populations and there is greater pollinator availability.

4.4.4 Populations at Beinn Heasgarnich.

Beinn Heasgarnich is the most genetically distinctive population; it has the only diagnostic AFLP fragment in the data set and also has the highest average pairwise Φst. It does not show a genetic affinity with surrounding *C. micacea* populations. This population may have been genetically isolated a comparatively longer time than the other upland populations. Morphologically, however it appears similar to other putative *C. micacea* populations (Figure 3). A chromosome count of 2n = 26 was made by Gill (1973) from plants of this population. So there is no reason to believe it is different from the populations at Ben Lawers and Ben an Dothaidh because of a different chromosome number.

4.4.5 Population at Little Kilrannoch

The Little Kilrannoch population was the only population sampled on serpentine rock, high in heavy metals. The soil was also freely draining, as opposed to the moist conditions in which upland *Cochlearia* are normally found. A study of *Cochlearia* at the Miekle Kilrannoch site (Nagy & Proctor 1997) found that the soil was low in potassium, high in nickel and magnesium, limiting plant growth and reproduction. The plants here were very small and pigmented with anthocyanin in wild populations, but reverted to 'typical' *C. pyrenaica* subsp. *alpina* morphology when grown in the greenhouse. It is not unusual to find

morphological (Baker & Dalby 1980) and life history (Nagy & Proctor 1997) differences between metalliferous populations and non-metalliferous populations in other plant species.

The cause of the low number of AFLP fragments derived from the Little Kilrannoch samples is uncertain. It is unlikely to be a diploid as other known populations have a restricted ecological niche next to base rich rivers and springs at moderate altitudes. In cultivation the morphology of the Little Kilrannoch population is most similar to that of the tetraploid populations (2n = 24). The population also had a lower number of polymorphic loci than populations from other sites. Both results could be explained by strong selection (Amos & Harwood 1998) or a population bottleneck leading to reduction in genetic diversity.

No major neutral genetic differentiation was detected between the population at Little Kilrannoch and other upland populations. There may have been undetected genetic changes. Sites with heavy metals place strong selective pressure on plant populations, even with gene flow from non-metalliferous populations (Lefébvre 1974, Jiménez-Ambriz *et al.* 2007). The plants at Little Kilrannoch are probably under strong selection, with the genetic variation in adult plants at Little Kilrannoch being a sub-sample of the potential genetic variation of immigrant genotypes.

4.4.6 The Ben Lawers site and *C. micacea*

Three options were considered at the Ben Lawers sites a) that the populations with variable pod length were a mixture of *C. micacea* and *C. pyrenaica* subsp *alpina*; b) that the two populations in different habitats were different species; c) that a narrow-podded variant with a discrete genetic grouping (i.e. *C. micacea*) could not be found.

There is no evidence for reproductive isolation or morphological differentiation between the two Ben Lawers populations. Slight differentiation was suggested by the number of private fragments and the chloroplast haplotype present in only one population. Separate clustering of the populations in the PCO plot does not refute the theory that there are two taxa at the site, but neither does it support it (particularly with such a low Φst value). The non-significant AMOVA result for partitioning of the variation between the populations is in stark contrast to the differentiation between the two populations at Port Geharia (chapter 3) which was ten times greater.

The habitats for the two populations are very different; one population was growing in a wet flush at around 800m. This site was relatively sheltered, with good water supply. The other population grows near the summit of Ben Lawers at around 1050m among a rich alpine flora, with longer annual snow cover. The two sites present different challenges to survival. Some of the private fragments between the populations may relate to adaptive changes in the genome.

Pod shape did not relate to genetic grouping, so the existence of a distinct long-podded form (*C. micacea*) is thrown into doubt. The morphology of reproductive structures is often preferred by taxonomists to characters derived from vegetative structures. However, the importance or significance of pod shape may have been overstated. Many Brassicaceae taxa show remarkable similarities in sequence data while showing drastically different fruit morphologies, and in other cases vice versa (Al-Shehbaz *et al.* 2006). Differences in fruit morphology could be misleading and therefore fruit characters should not be over-emphasised at the expense of other characters (Al-Shehbaz *et al.* 2006). Studies on *Brassica napus* (Chay & Thurling 1989), a relative of *Cochlearia*, have revealed that pod length is controlled by only a few genes and different forms are produced by minor allele changes.

If *C. micacea* can be reliably confirmed by the 2n = 26 chromosome count, this appears to be uncoupled from the described *C. micacea* morphology. The distribution of the 2n = 26 (*C. micacea*) karyotype is not fully known. The plants that grow near the Ben Lawers population at Meall nan Tarmachan have a confirmed chromosome count of 2n = 26 (Gill 1973), although these plants have short rounded pods and some had large leaves, up to 2 cm across. Scandinavian taxonomists also suggested that accessory chromosomes in 2n = 24 taxa were mistakenly counted as an extra pair of chromosomes resulting in the 2n = 26 count (Nordal & Laane 1990). Even if the extra chromosome pairs were correctly observed, if there are no accompanying changes in habitat preferences and fertility, the aneuploidy may be of no wider significance and could simply be viewed as a polymorphism.

Cochlearia micacea appears at most to be a dwarf alpine form of *C. pyrenaica* subsp. *alpina* that may have undergone parallel adaptive changes at different sites in response to the harsh environment of the mountain summits. *Cochlearia micacea* has a thick tap root; this tendency to store resources under ground is common adaptation to higher altitude life, as is the low growth form and vegetative reproduction and a perennial life span, all distinguishing features of *C. micacea* (Billings 1974).

4.5 Conclusions

As with the coastal populations, there is a considerable range of morphological variation within and between populations, but this variation does not form a coherent pattern at higher level groupings. The upland populations had a very small amount of chloroplast variation and it was not taxonomically informative. The chloroplast variation that was present was not distributed in a fashion coincident with previous taxonomic hypotheses.

There was no grouping of neutral genetic variation or morphological characters by described taxonomic groups. In particular no grouping that could be ascribed to *C. micacea* was found at the type location for this taxon, Ben Lawers. Caution should be exercised in making taxonomic recommendations almost entirely based on molecular marker evidence. However, the continuing difficulties surrounding *C. micacea* identification and its lack of clear ecological differentiation, add weight to the suggestion that it does not exist as a distinct taxon. *C. pyrenaica* subsp. *pyrenaica* populations were insufficiently sampled to draw firm conclusions regarding its status.

Cochlearia micacea could merit conservation interest as an adaptive form of higher altitudes. If this was the case, it would have lower conservation status than it currently has, because this adaptive form seems to have been generated at multiple sites, indicating that it could arise again given suitable conditions. However, the same difficulties in delimitating the group would remain. The main threat to higher altitude communities is climate change. Plants can respond to temperature increase by moving to a higher altitude; however plants that already live at the summit will not be able to respond in this way. If *C. micacea* is at most high altitude growth form and is lost as a result of climate change, the loss to biodiversity would be minimal.

5. The upland and coastal populations: separate lineages or ecotypes?

Abstract

Cochlearia grows in a range of habitats in Britain. These can be split broadly into two types: coastal and upland. Having established in previous chapters that we cannot identify discrete taxonomic clusters within the coastal and upland groups, we will now examine the hypothesis that there are two separate genetic groupings: in the uplands and around the coast. This chapter will also infer which of two evolutionary scenarios is most likely to have led to the occurrence of upland and coastal morpho-types in different regions 1) that upland and coastal plants have formed multiple times in different regions in response to the environment or 2) that plants from the two groups arose separately once and spread to all suitable habitats. Five pairs of populations were sampled. In each of the five regions a population from the coast was sampled and a population from the uplands was sampled. Then variation in AFLP fragments among and between the five pairs was used to test which evolutionary scenario best fit the data. There was a weak relationship between proximity of the regional pairs and their genetic similarity. The upland and coastal populations did not cluster separately, and so it is most likely that the coastal and upland morphological types formed multiple times in different regions.

5.1 Introduction

Cochlearia of the uplands and coasts have been consistently treated as separate groupings (Clapham *et al.* 1981, Nordal & Laane 1996). It is unknown whether plants from the two habitat types originate from two different lineages. The evolutionary schemes proposed by Elkington (1984) and Koch *et al.* (1998), both suggest modern *Cochlearia* originate from ancestral diploids of a similar type to *C. aestuaria* and *C. pyrenaica*. The appearance of tetraploids (2n = 24) with greater genomic flexibility than diploids may have facilitated the movement of *Cochlearia* from a restricted range of diploid habitats, beside base rich springs and rivers at moderate altitudes (now found in Northern England and Skye), to a much broader range of upland and coastal habitats (Gill *et al.* 1978). It is not known whether the current British populations of *C. officinalis* s.l. originate only from the plants that survived in Britain during the last ice age, or whether they have been supplemented by colonisers from the rest of Europe.

5.1.1 The challenges faced in upland and coastal habitats

Plants face quite different challenges in upland habitats compared with coastal habitats. Inland-upland populations experience more extremes of temperature the coastal populations because of the moderating effect the sea has on temperature. Upland plants have a much shorter growing season and those plants at higher altitudes may spend part of the year under snow. The amount of insect herbivory may also vary between populations at different altitudes (Galen *et al.* 1991). Coastal plants experience considerable osmotic stress due to high NaCl concentrations and/or free draining substrates (Rozema *et al.* 1985). In contrast, upland *Cochlearia* populations are normally associated with a constant supply of water, and so must adapt to water-logging, rather than water-stress. There are also differences in nutrient availability, in a Scandinavian study of ecotypic variation in *Cochlearia*, the upland habitats were found to be considerably higher in calcium and potassium, but in some cases lower in nitrogen than the coastal habitats (Nordal & Stabbetorp 1990). Within the two broad habitats types there may be a range of different niches. A coastal plant growing on a bird cliff, may occupy a habitat which is physiologically more similar to an upland base rich flush than to an estuarine site.

5.1.2 Adaptive response to a disjunct upland-coastal distribution in other plant species

Physiological changes that permit survival in varied habitats have also been found in many other plant groups (Linhart & Grant 1996, Rozema *et al.* 1985). More specifically, there are other plant groups which share a disjunct coastal-upland distribution pattern with *Cochlearia* and are often found as associated species with *Cochlearia* populations. These species include *Armeria maritima* (Woodal & Dale 1993), *Plantago maritima* (Gregor 1938), *Silene maritima* (Dalby & Baker 1980) and *Agrostis solonifera* (Kik *et al.* 1990). Studies on these plants using reciprocal transplant experiments have revealed a range of adaptations among these plants to suit different habitats. The most typical hereditary changes for these plants are growth form, leaf shape and plant size (Dalby & Baker 1980, Gregor 1938, Woodal & Dale 1993). In addition changes in life history can occur e.g. changes seed production and life span (Kik *et al.* 1990).

5.1.3 Adaptive response to upland and coastal distribution in the genus *Cochlearia*

Some evidence has been gathered for differential adaptation between the upland and coastal ecotypes in *Cochlearia*. *C. anglica* - an estuarine taxon - germinated much better in the dark

and in the presence of a high NaCl concentration than *C. pyrenaica* - a mountain taxon (Pegtel 1999). Seeds of saltmarsh or estuarine plants may become buried in mud and therefore need to germinate in the dark (Rosema *et al.* 1985), whereas mountain individuals need to delay germination until after the snow has melted and so will tend to germinate in response to light (Billings 1974). Nordal & Laane (1990) found that bud development in upland plants commenced earlier than in coastal plants and that buds were already fully formed under the snow. The buds are ready to flower as soon as the snow melts as an adaptation to short growing season in the uplands. Increased ploidy level is associated with increased salt tolerance (Pegtel 1999). All the coastal ecotypes can tolerate and germinate in higher NaCl concentrations than upland ecotypes (Pegtel 1999).

5.1.4 Morphological character differences between upland and coastal *Cochlearia* populations

There are some differences in morphological characters between the upland and coastal ecotypes. *Cochlearia* plants at higher altitudes have adaptations to alpine life e.g. perennial tap roots, compact growth form, small, waxy leaves and some vegetative reproduction (Billings 1974, Gill *et al.* 1978, pers. obs. 2004, 2005). Coastal plants may also have a compact form in heavily grazed situations or where they are exposed to wave action. Many of the morphological differences between populations in different habitat types could be ascribed to differential environmental pressures on development. Although, as we have seen in previous chapters, characters such as growth form and leaf shape are maintained when plants are grown under standardised conditions. These observations are in agreement with the work on *Cochlearia* from Scandinavia and the Netherlands (Pegtel 1999, Nordal & Laane 1996, Nordal & Stabbetorp 1990).

5.1.5 Adaptive change and neutral genetic markers

A great deal of evidence for local adaptation has been discovered using common garden and transplant studies (Linhart & Grant 1996). However, similar locally adapted ecotypes from different places often don't correspond to neutral genetic groupings (Vijverberg *et al.* 2000, Schmidt-Lebuhn 2007, Hedrén *et al.* 2001). Almost every aspect of plant morphology and life history can be affected by local selection that creates different forms in different habitats. These changes often do not effect neutral genetic variation. Some phenotypic differences in plants from different habitats may result from inherent plasticity and so no genetic changes will have occurred (Agrawal 2001). Adaptive divergence may also occur at a small number of loci, or be the result of allele frequency differences over many loci, so again major genetic

change will not be observed. Adaptation to new habitats may indirectly reduce gene flow and consequently lead to differentiation. Examples of this may be changes in flowering time, breeding system or pollinator species (Billings 1974, Macnair 1989). A similar genetic pattern will occur if there is very low survivorship among migrant genotypes**.** Both of these scenarios lead to genetic differentiation among adult populations in different habitats

5.1.6 Ecotypes or lineages

When populations of closely related species are morphologically or physiologically differentiated according to the habitat in which they grow, they may represent separate lineages or locally adapted ecotypes. In the first scenario, 'the separate lineage scenario', lineages originated separately and then spread to similar habitats. This scenario has been proposed for the fen and sand dune forms of *Liparis loeselii* (Pillion *et al.* 2007). The maintenance of two distinct lineages implies that they are reproductively isolated from one another or that they rarely come into contact. In the second scenario, 'the ecotype scenario', the same morphological and ecological type has evolved in parallel many times in response to the same habitat. This scenario has been proposed for *Microseries* in Australia and New Zealand (Vijerberg *et al.* 2000), the allotetraploid *Dactylorhiza* of Europe (Hédren *et al.* 2001) and *Minthostachys* (Schmidt-Lebuhn 2007). If there has been extensive gene flow between formerly divergent lineages, then it is difficult to distinguish which of the two scenarios has occurred.

5.1.7 Implications for conservation and management

If there are putative rare lineages (or ecotypes), as there are in *Cochlearia* (see Chapters 3 & 4), then distinguishing between these two scenarios becomes vital for conservation and management. If an ecological and morphological type has arisen multiple times, then that morphological type has the potential to arise again through the same processes from the same progenitors. However, if the morphological and ecological groupings are independent lineages, these lineages may come from a single event that may not occur again

5.1.8 Questions

Are populations more genetically similar to other populations from the same habitat irrespective of geography, or are they more similar to neighbouring populations found in a different habitat?

5.1.9 Approach

The purpose of this chapter is first to discover whether the ecotype scenario or the separate lineage scenario best explains the patterns of variation in *Cochlearia*. The second purpose is, to define the relationship between the populations occupying the two habitat types and infer the modes of colonisation and diversification that have occurred. Taxonomic classifications of populations were not attempted, because previous chapters indicate that they do not constitute neutral genetic groupings. Pairs of populations of *Cochlearia* from the uplands and the coast have been sampled as close together as possible from different regions. The relationship between habitat type, geographic distance and genetic grouping was analysed using AFLP markers. The amount of gene flow or ancestral similarity between populations was estimated by looking at patterns of shared and private AFLP fragments. If the coast and upland plants are two separate lineages then they will be more similar to plants from the same habitat than to their regional pairs. If the ecotype scenario better explains the data then the upland and mountain plants will not form two separate groups, but will be intermixed. There may be a link between geographical and genetic distance if the ecotypes theory best explains the data, but this is not necessarily the case.

5.2 Methods

5.2.1 Sampling

Between 10 and 15 plant samples were collected from two populations in each of five regions. The regions were North-West Scotland; South-West Scotland; East Scotland; Northern England and Wales. Within each region an upland population and a coastal population were sampled. The distance between populations within pairs differed between 9km and 133km apart depending on the availability of suitable proximal populations. The distribution of the regional pairs is shown in Figure 5.1. The locations of the populations along with a list of associated species are also shown Table 5.1.

5.2.2 AFLP marker generation

The AFLP variation was scored as described in Chapter 2.

5.2.3 AFLP analysis

The AFLP data were handled and analysed as in Chapter 3. Individuals were scored for variation at AFLP marker loci. AFLPs were used to detect differentiation between regions and habitats. The following groupings were used for PCO analysis: region, habitat and population. For the AMOVA analysis the groupings individuals and populations were nested within regions and individuals and populations nested within upland and coastal groupings. The numbers of shared and private fragments were used to detect amounts of gene flow between upland and coastal populations. As in Chapter 3, a Mantel test was used to determine whether there was a link between genetic and geographical distance.

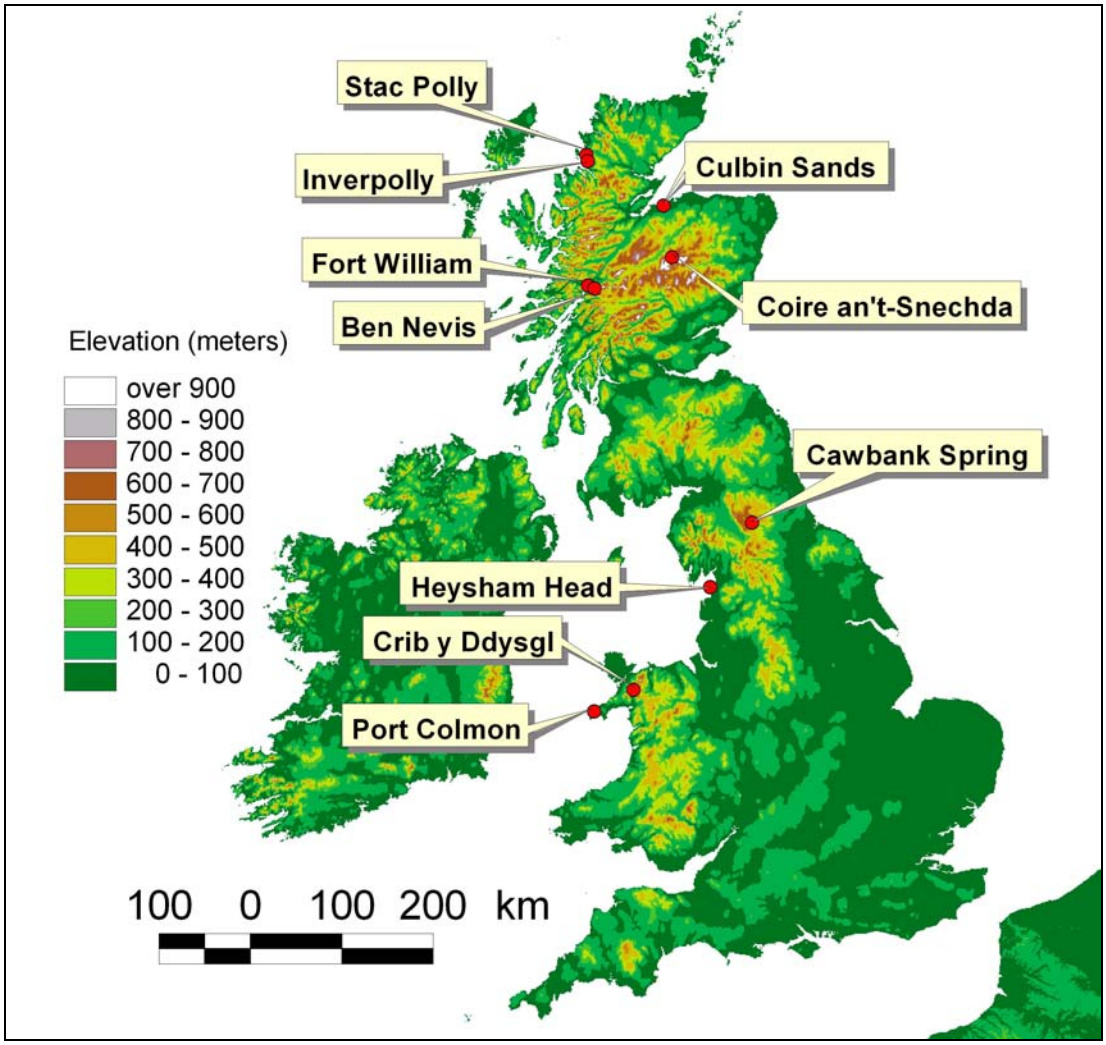

Figure 5.1: A map showing the locations of populations sampled for a study of genetic variation between upland and coastal populations. The regions and pairs are**: N England:** Cawbank spring and Heysham Head; **Wales:** Crib y Ddysgl and Port Colmon; **E Scotland**: Culbin sands and Coire an't-snechda; **NW Scotland**: Stac Polly and Inverpolly; **SW Scotland**: Ben Nevis and Fort William.

Site locations (upland or coast)	UK national grid reference	Habitat and associated species	No. of samples for AFLP analysis
N. England (populations 80km apart)			
Cawbank spring* (U)	NY/856.279	Upland: Base-rich upland spring. Associated species not recorded.	**10**
Heysham Head (C)	SD/407.612	Coastal: sandstone cliffs, *Taraxacum officinale, Rumex crispus, Festuca rubra, Plantago maritima, Hypochaeris radicata, Asplenium marinum*	**10**
Wales (populations 46km apart)			
Crib y Ddsgyl, (U)	SH/605.555	Upland: base-rich flushed crags *Poa alpina, Chrysosplenium oppositifolium, Oxyria digyna, Deschampsia cespitosa, Cystopteris fragilis, Festuca ovina, Sedum rosea, Ranunculus acris,* Bryophytes, *Saxifraga stellaris,Saxifraga oppositifolia,*	**10**
Porth Colmon (C)	SH/194.342	Coastal: on low cliffs and slipway *Plantago coronopus, Festuca rubra, Tripleurospermum maritima, Armeria maritima, Cochlearia danica, Apium nodiflorum, Agrostis stolonifera, Oenanthe crocata*	**10**
East Scotland (populations 133km apart)			
Coire an t-Sneachda (U)	NH/996.032	Upland: scree filled flush, granite *Saxifraga rivularis, Deschampsia cespitosa, Oxyria digyna, Alchemilla alpina, Epilobium anagallidifolium, Rumex acetosa, Poa alpina, Stellaria uliginosa*	**10**
Culbin Sands (C)	NH/901.577	Coastal: upper saltmarsh *Plantago maritima, Glaux maritima, Armeria maritima, Aster tripolium, Puccinella maritima, Salicornia agg,*	**10**
NW Scotland (populations 32km apart)			
Stac Polly (U)	NC/111.103	Upland: wet S-facing granite cliff *Sedum rosea, Luzula sylvatica, Succisa pratensis, Primula vulgaris, Angelica sylvestris, Ranunculus acris, Epilobium anagallidifolium, Cirsium palustre*	**9**
Inverpolly (C)	NB/943.683	Coastal: mixed saltmarsh and shingle *Triglochin palustre, Triglochin maritima, Juncus gerardi, Armeria maritima, Plantago martima, Festuca rubra*	**9**
SW Scotland (populations 9km apart)			
Fort William (C)	NN/087.764	Coastal: shingle beach and sea wall *Taraxacum officinale, Trifolium repens, Plantago coronopus, Spergularia media*	**8**
Ben Nevis(U)	NN/161.721	Upland: Scree filled gully Bryophytes*, Oxyria digyna*	**9**

Table 5.1: Sampled population locations & associated plant species (*possible diploid 2n = 12).

5.3 Results

5.3.1 AFLP marker frequency distribution.

AFLP analysis produced 285 polymorphic scorable fragments. There were no private high frequency fragments for regional groups (see Table 5.2). The Cawbank spring population produced the lowest average marker number per sample with 37.3. The Ben Nevis population produced the highest average marker number per sample with 59.0 (Table 5.2). The percentage of fragments polymorphic at the 5% level was between 36.7% and 48.1% in all populations except Cawbank spring which had a considerably lower percentage of 31.8%. Ben Nevis, Stac Polly, Inverpolly, Heysham Head and Crib y Ddysgl had between one and two high frequency private fragments (Table 5.2). The populations at Crib y Ddysgl and Inverpolly had the highest number of private fragments overall.

When the AFLP marker distribution between upland and coast and between each regional pair were compared (see Table 5.3), there was only one high frequency private fragment present in 38% of upland samples, absent from coastal populations. The vast majority of the fragments were shared between coastal and upland populations (191 shared fragments). Ben Nevis and Fort William shared a much higher proportion of fragments than the other regional pairs. There are many high frequency fragments that were not shared between Inverpolly and Stac Polly.

5.3.2 ANOVA of marker distribution.

There was significant variation in marker number among populations, but none among regions (Table 5.4) or upland and coastal groupings (Table 5.5).

Upland and Coast	Mean average fragment number	% polymorphic at 5% level	Private fragments	Diagnostic fragments	Present in 50% or more	Singletons	% singletons
England	45.0		3	0	0		0
CawbankSpring (U)	37.3	31.8	5	0	0	5	100
HeyshamHead (C)	52.7	40.6	5	0	2	2	40
Wales	54.5		1	0	0	1	100
CribyDysgl (U)	53.2	48.1	11	0	1	9	82
PorthColmon (C)	56.0	43.1	8	0	0	8	100
E. Scotland	53.7		2	0	0	0	0
Coirean'tSnechda (U)	51.3	36.7	1	0	0	1	100
CulbinSands (C)	56.2	46.6	9	0	0	7	78
NW Scotland	53.5		1	0	0	1	100
StacPolly (U)	58.6	37.5	7	0	1	5	71
Inverpolly (C)	53.3	45.9	12	0	2	9	75
SW Scotland	56.5		1	0	0	0	0
FortWilliam (C)	53.75	47.3	3	0	0	3	100
BenNevis (U)	59.0	40.6	2	0	1	1	50

Table 5.2: Mean average fragment number and distribution of private fragments among populations and among regions of sampled upland and coastal populations. U = upland populations and C = coastal population

Region/Populations	Private fragments	Private Present in >50%	Singletons	Shared fragments
N. England				
Heysham Head (C)	48	7	26	43
Cawbank spring (U)	39	2	24	
Wales				
Porth Colmon (C)	55	1	35	52
Crib y Ddysgl (U)	66	6	30	
E Scotland				
Coire an t-Sneachda (U)	34	3	5	55
Culbin sands (C)	54	1	15	
NW Scotland				
Inverpolly (C)	27	7	33	64
Stac Polly (U)	49	11	20	
SW Scotland				
Ben Nevis (U)	31	4	15	84
Fort William (C)	50	2	34	
Upland	38	1	18	191
Coast	53	0	28	

Table 5.3: Shared and private fragments between regional pairs and between habitats of the sampled upland and coastal *Cochlearia* populations. U = upland populations and C = coastal populations.

Source	DF	MS
Region	4	409.95
Between populations within region	5	317.96**
Within population	85	76.06
Total	94	

Table 5.4: GLM nested ANOVA of AFLP marker number for *Cochlearia* populations sampled from different regions and habitats across Britain. Populations are nested within regions. (* = P value < 0.05, ** = P value < 0.01, *** = P value 0.001)

Source	DF	MS
Habitat	1	45.20
Between populations within habitat	8	398.38***
Within population	85	76.06
Total	94	

Table 5.5: GLM nested ANOVA of AFLP marker number for *Cochlearia* populations sampled from different regions and habitats across Britain. Populations are nested within habitats.
(* = P value < 0.05, ** = P value < 0.01, *** = P value 0.001).

5.3.3 Principal co-ordinates analysis

The first two axes of the PCO plot (Figure 5.5) only accounted for a small proportion of the variation (axis 1 = 9.1% and axis 2 = 6.4%). Populations did not group into upland and coastal groups. The strongest groupings in the data were by population, only two of the five regional pairs grouped by geography. These were Crib y Ddsgyl and Porth Colmon in Wales and Ben Nevis and Fort William in N.W Scotland. Stac Polly clusters far from its regional neighbour Inverpolly and the other populations. The upland and coastal pairs from each region clustered in mutually exclusive groups to each other when plotted alone using PCO analysis (not shown).

5.3.4 AMOVA

AMOVA analyses of the AFLP data showed that there was significant genetic variation among populations, and regions (Table 5.6), but no significant variation between habitats (Table 5.7). The population differentiation statistic derived from AMOVA ($\Phi st = 0.29$) indicated that differentiation among populations was substantial. The differentiation between regions was marginally significant and accounted for a small amount of the variation (>4%). There may have been a weak relationship between genetic grouping and regional grouping. The PCO plot showed Ben Nevis and Fort William, Port Colmon and Crib y Ddysgl regional paired populations cluster together, but summary statistics hid this pattern.

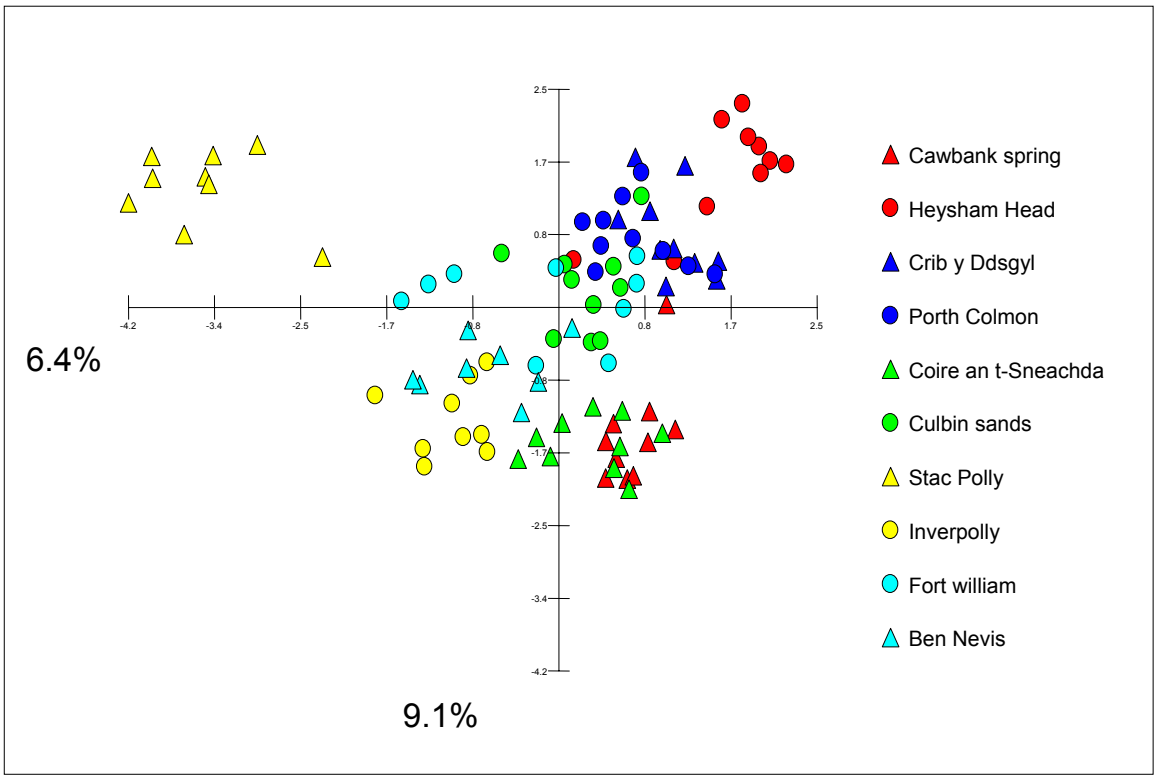

Figure 5.2: PCO plot showing phenetic relationships between upland (triangle) and coastal (circle) populations of *Cochlearia* from different regions in the British Isles based on AFLP marker variation converted to Jaccards similarity co-efficient.

Regions colour code: Red = Northern England, Dark Blue = Wales, green = Eastern Scotland, Yellow = N.W Scotland, light blue = N.W. Scotland. The % variation in the data set explained by each axis is given on the axis labels.

Source of Variation	Degrees of freedom	Sum of squares	Variance components	% of variation	P values
Among regions	4	380.67	0.9025	3.67	0.055
Among population within regions	5	390.15	6.3959	25.99	<0.001
within populations	85	1471.13	17.3075	70.34	<0.001
Total	94	2241.96	24.6058		

Table 5.6: AMOVA results for AFLP variation for upland and coastal *Cochlearia* populations sampled between regions across Britain. Populations are nested within regions.

Source of Variation	Degrees of freedom	Sum of squares	Variance components	% of variation	P values
Among habitats	1	87.192	0.02792	0.11	0.471
Among pops within habitats	8	683.633	7.18213	29.29	<0.001
Within populations	85	1471.133	17.30745	70.59	
Total	94	2241.958	24.51749		

Table 5.7: AMOVA results for AFLP variation for upland and coastal *Cochlearia* populations sampled between coastal and upland habitats across Britain. Populations are nested within habitats.

5.3.5 Φst and mantel test

Overall the Φst values (Table 5.8) were higher in this dataset than in the previous two, the majority of values were Φst >0.25 and the average Φst was 0.28, as opposed to Φ = 0.17 and Φ = 0.18 for the coast and mountain datasets respectively. All of the populations showed considerable Φst differentiation from all other populations, regardless of how closely geographically situated they were. With the exception of Stac Polly, the patterns of differentiation between populations were fairly homogenous, with each showing more or less equal level of differentiation from all other populations regardless of their location or habitat. The population at Stac Polly showed a striking degree of differentiation from all other populations (Stac Polly mean population Φst = 0.41, the other populations have mean values ranging between Φst = 0.18 and 0.31 which was in accordance with its position on the PCO plot. The level of differentiation between Stac Polly and its regional neighbour Inverpolly re-enforces the results of the marker distribution analysis and the PCO plot. Ben Nevis showed lower differentiation from the Fort William population compared with the other populations, also in accordance with their position on the PCO plot. Crib y Ddysgl and Porth Colmon show a considerable degree of differentiation, despite being one of the region pairs that grouped most closely together in the PCO plot. The Mantel test showed no significant relationship (R^2 = 0.099) between genetic and geographical distance.

	Cawbank	Heysham Head	Crib y Ddsgyl	Porth Colmon	Coire ansnechda	Culbinsands	Stac Polly	Inverpolly	Fort William	Ben Nevis
Cawbank	0									
Heysham Head	0.369	0.000								
Crib y Ddsgyl	0.253	0.216	0.000							
Porth Colmon	0.314	0.234	0.201	0.000						
Coire an t-snechda	0.260	0.347	0.251	0.287	0.000					
Culbinsands	0.282	0.250	0.176	0.175	0.228	0.000				
Stac Polly	0.501	0.495	0.404	0.420	0.458	0.373	0.000			
Inverpolly	0.327	0.388	0.301	0.316	0.290	0.286	0.393	0.000		
Fort William	0.214	0.193	0.135	0.126	0.185	0.095	0.299	0.187	0.000	
Ben Nevis	0.285	0.346	0.274	0.297	0.265	0.213	0.388	0.293	0.161	0.000
Mean population average	0.312	0.315	0.246	0.263	0.285	0.231	0.414	0.309	0.177	0.280

Table 5.8: matrix of Pairwise Φst differentiation based on AFLP data, between ten populations sampled from the uplands and coast. All Φst values significant to P = 0.05 level.

5.4 Discussion

5.4.1 Overall trends in the upland and coastal dataset

Populations from the same habitat type, mountain or upland are not more similar to other populations from the same habitat than they are to neighbouring populations from different habitat types. So the hypothesis that the similar morphological types in similar habitats are of the same lineage does not fit the data. The theory that morphotypes and ecotypes are created by local adaptation is the most likely explanation.

There was no clear correlation between genetic and geographical distances among populations according to the Mantel test. This is not an unexpected result, a weak or non-existent relationship between geographical separation of populations and genetic similarity has been noted in studies on other colonising plant species (Jørgensen & Mauricio 2004, Schonswetter *et al.* 2006b, Después *et al.* 2002). The lack of structure with respect to geography suggests that the Φst values are indicative of a more complex set of factors than simple genetic isolation by distance. The results of the Mantel test should not be used in isolation to infer the presence or absence of isolation by distance. Simple correlation tests of linear geographical distances do not take landscape features into account and these may change rates of gene flow e.g. gene flow facilitated by water around the coasts, but obstructed by mountains in mountainous regions.

5.4.2 Gene flow between populations

The Φst values derived in this study (0.18-0.43) are generally higher than the average for out-crossing, insect pollinated plants (~0.20 - from allozyme data, Hamrick & Godt 1990), but lower than the average values for inbreeding taxa (Φst = 0.50-0.60 (Bussel 1999). The low observed seed set in many populations makes a shift to selfing unlikely, although this has not been empirically tested. The most likely explanation is that the topology and lack of long distance pollinators results in low levels of gene flow between populations.

5.4.3 Gene flow between regional population pairs

The paired populations all have reasonably high Φst values (Φst = 0.161-0.393) between them indicating low levels of gene flow between upland and coast. However, the Φst values are still within the region of Φst = 0.2-0.3, if no other factors are taken into account when interpreting Φst except gene flow this equates to almost one migrant per generation, enough to prevent substantial differentiation by drift (Spieth 1974). Opinions vary about the exact levels of migration required to prevent drift, although it appears that there is enough gene

flow to prevent inevitable differentiation by drift in *Cochlearia*. However, if the *Cochlearia* populations have come from a common source since the last glaciation then Φst values are likely to be reduced by the presence of many common ancestral markers. Therefore real levels of gene flow could be lower than the Φst values suggest.

There was a trend toward greater similarity between regional pairs that were closer together. Fort William and Ben Nevis (9km apart), Crib y Ddysgl and Port Colmon (46km apart) had interleaved clusters in the PCO plot of populations. They also had fairly low Φst values compared with other regional pairs: 0.161 and 0.201 respectively. The regional pair of populations Stac Polly and Inverpolly were 31km apart, but were clustered separately in the PCO plot and so did not support this trend. Stac Polly was highly differentiated from all other populations (as will be discussed in more detail in section 5.4.6). Cawbank spring and Heysham head (80km apart and in a different river catchment) were separated in the PCO plot and had a pairwise differentiation value of Φst = 0.369. Culbin sands and Coire an't-Snechda were further apart still (133km), and were also genetically dissimilar (Φst = 0.228). Although overall patterns of genetic similarity did not produce a pattern of isolation by distance, it appears that populations that are geographically close are more genetically similar than those further from each other.

There are a number of ways that gene flow could occur between the uplands and the coast, particularly where they are close to each other. Movement of seeds or plants from upland to coast via streams is probable, as mountain-type plants are sometimes found beside lowland rivers (pers. com. Rich 2007). Transportation of seeds between the coast and uplands by birds may also occur (Howe & Smallwood 1982), although this has not been directly observed in *Cochlearia*. Flowering times for all British *Cochlearia officinalis* s.l. taxa overlap, because there is a long flowering season from May to September (Dalby 1991). Gene flow by pollen could occur between populations where there was a suitable vector. The average pairwise Φst between coastal populations (Φst = 0.23) is much lower than between upland populations (Φst = 0.33). As discussed in Chapter 4, this is thought to be because gene flow can occur more easily between the coastal habitats than upland habitats, although this theory was only weakly supported by the results from chapters 3 and 4.

5.4.4 Post glacial colonisation and adaptation

It is unclear whether the relict populations were the progenitors for all of the UK populations, or whether their descendants are mixed with colonisers from continental

Europe. We know that upland *Cochlearia* existed in Southern England at the height of the last glaciation (Godwin 1964, Godwin 1975 in Lang 1995). They are also a cold tolerant, evergreen species, so they could have been on the leading edge of plant re-colonisation after glacial retreat (Nordal & Laane 1996, Moreau *et al.* 2005). Some studies of post glacial colonisation in other species have found separate lineages indicating diverse refugial origins (Hewitt 2004, Lambracht *et al.* 2006). Although diverse refugial sources cannot be ruled out for the British *Cochlearia,* there is nothing in the data to suggest the modern assemblage comes from more than one source. Late glacial conditions were probably favourable to *Cochlearia* (Moreau *et al.* 2005, Godwin 1964). Therefore, populations of *Cochlearia* may have been more widespread with greater levels of gene flow between them than in the present day. Genetic differentiation between populations from diverse sources may have been obscured by historical gene flow.

5.4.5 Evidence for local adaptation.

There are considerable differences in the environmental challenges presented by upland and coastal habitats. Differential selective pressures resulting in adaptation are almost inevitable. The changes that result from these selective pressures cannot be identified using the available data. The wild populations cultivated in the green house for Chapter 4, showed that the upland morphology (compact, small leaved plants) was maintained in standard conditions. There is indirect evidence that higher altitude *Cochlearia* are less well adapted to insect herbivory. Plants from Beinn Heasgarnich and Ben Lawers were heavily attacked by greenfly (*Aphis sp*.) in the greenhouse, whereas the plants collected from the coast were unaffected. Brassicaceae contain glucosinolate (mustard oil) as a pest deterrent and this substance has been found in three sampled populations (*C. officinalis* subsp. *scotica*, *C. atlantica* and *C. micacea*), that were sent to collaborators (Dauvergne *et al.* 2006). The relative quantities of the substance are not known, but differing quantities of this substance from different ecotypes could explain differences in pest response. Differential adaptation to pests at different altitudes has also been identified in *Polemonium viscosium* ecotypes from different altitudes (Galen *et al.* 1991).

5.4.6 Populations at Stac Polly and Cawbank spring.

The Stac Polly population is particularly distinctive, clustering far from the other samples in the PCO plot and with high levels of Φst differentiation from the other populations. Stac Polly was an unusual population because morphologically it appeared to be a *C. officinalis* s.s., but it was growing in a mountain flush at 450m. The reason for the genetic and

morphological distinctiveness is not known. Cawbank spring has significantly fewer fragments than the other populations. This may be because this population had a small number of founders or has experienced a genetic bottleneck. The most likely explanation is that heterozygosity has been lost by genetic drift in this small isolated population.

5.5 Conclusions

The upland and coastal species do not form two separate lineages. The genetic variation within *Cochlearia* is unstructured beyond the population level, with no strong indications of grouping by taxonomic classification or by geographical location. Two of the regional population pairs that were geographically close, also showed greater genetic similarity compared with other populations.

The low level of structure and variation in the genetic data make it difficult to infer the mode of colonisation in *Cochlearia*. However, the lack of structure and variation is itself a symptom of the processes that have occurred. As we have found in the previous chapters, the overall picture is of a rapid unstructured colonisation by *Cochlearia* from one or few closely related sources, followed by local adaptation to different habitats. The process of adaptation has probably been enhanced by subsequent low levels of gene flow. So the 'ecotype scenario' best fits the data, although we did not find a close genetic relationship between regional pairs as expected for this scenario. The use of uncharacterised genetic data has highlighted the lack of structure in neutral genetic variation, but on a UK scale there is little more to be gained from this approach. Reciprocal transplants between upland and coastal populations would indicate how important local adaptation is to survival in *Cochlearia* populations. Hybrids resulting from inter-population crosses could also be assessed for survival in the same way.

6. Discussion: the causes of morphological and ecological variation in British *Cochlearia* and its consequences for taxonomy and conservation

6.1 Introduction

The patterns of neutral genetic variation within the *C. officinalis* s.l. species complex in the UK have been clarified using AFLP markers combined with morphological characters. The existence of the putative endemic taxa *C. atlantica*, *C. officinalis* subsp. *scotica* and *C. micacea* as distinct groups will be considered. The overall patterns of variation will be clarified, and evolutionary scenarios put forward. Then the taxonomic and conservation implications of these results are discussed.

6.1.1 Overall patterns of variation

A common feature of the morphological, chloroplast and AFLP data presented in this thesis is the lack of structure above the population level. Distinctive population morphologies are often maintained in cultivation, so there is a genetic element to the morphological variation observed between natural populations. The chloroplast variation was very limited and not informative. This is in accordance with a much larger study that showed very low chloroplast variation across Europe (Koch *et al.*1998). The fairly high differentiation (Φst = 0.15- 0.36) between populations indicates fairly low levels of gene flow. The AFLP marker variation showed no indication of grouping by taxonomic classification. There were only weak indications that genetic similarity was influenced by geography e.g. the greater differentiation of isolated coastal populations (Section 3.4.2); the genetically similarities between two regional pairs (Section 5.4.3). There is no evidence for distinct lineages between the different *Cochlearia officinalis* s.l. taxa or ecotypes. The predominant generator of variation in *Cochlearia* appears to be local genetic ecotypic adaptation to different habitats. The low level of geographical structure and lack of taxonomic structure in the data have been found in other taxa and are associated with recent, unstructured post glacial colonisation or introduction (Després *et al.* 2002, Jørgensen & Mauricio 2004). This can result from processes such as: strong selection (Ehrich *et al.* 2007), small source populations and/or long distance founding events (Després 2002, Gaudeul *et al.* 2000).

6.2 Post glacial colonisation and adaptation

6.2.1 Source populations and modes of colonisation.

The latest information on glacial extent shows that there were significant ice-free areas in the south of England (Brochmann *et al*. 2003). Evidence from glacial and post-glacial deposits suggests that *Cochlearia* populations survived in refugia there, then spread rapidly across the UK during glacial retreat. *Cochlearia* macrofossils have been found on at least three occasions in Late Weichselian (during the height of the last glaciation) and late glacial deposits (a time of glacial retreat). The macrofossils have been recorded as part of refugial and pioneer vegetation communities from deposits in the south east of England at Colney Heath, the Lea Valley and Norfolk (West *et al.* 1974); and also from South West England (Godwin 1964, Reid 1949, Godwin 1975 in Lang 1995). *Cochlearia* are cold tolerant, with a modern distribution well into the Arctic Circle, so their persistence in refugia in Southern England at the height of the last glaciation is certainly feasible. The late glacial conditions would have been suitable for the spread of refugial *Cochlearia* populations. A study of contemporary vegetation colonisation after retreating glaciers in Greenland shows that *Cochlearia* colonise areas within 30 years of glacial retreat, and are one of the species that features heavily in the pioneer communities (Moreau *et al.* 2005).

Whether or not *Cochlearia* populations re-colonised from one source or diverse sources is difficult to deduce from the available information. Plants may have colonised from continental European, as well as from British refugia. In some plant species where refugial populations are sufficiently differentiated from each other then distinct lineages should be apparent in contemporary populations (Schonswetter *et al.* 2003, Hewitt 2000). However, there was not enough signal or variation in the AFLP or chloroplast data for *Cochlearia* to infer numbers or locations of source populations. Cochlearia are cold tolerant and were not confined to small Southern European refugia during the last ice age as many less hardy species were. Therefore genetic patterns (e.g. very low diversity, highly differentiated lineages) caused by confinement to small isolated populations and long distance dispersal are not likely to be found in *Cochlearia*. In addition, strong patterns of reticulation caused by genetically differentiated lineages re-mixing may be less prominent among hardy species like *Cochlearia*.

Cochlearia pyrenaica subsp. *pyrenaica* (ancestral diploid) is found in base rich springs with low levels of plant competition. These populations are thought to be the remnants of the first

northward colonisation as the glaciers retreated (Gill *et al*. 1978). The base rich springs and areas of bird nesting sites/bird cliffs are the first areas to be colonised by *Cochlearia* in contemporary studies of glacial retreat as most other areas are lacking in nutrients or organic content (Russell *et al.* 1940). The time at which the first polyploids appeared is unknown, although was thought to be a post-glacial event (Koch *et al.* 1998). An increase in ploidy level through autopolyploidy is associated with increased ecological tolerances, including NaCl tolerance (Brochmann *et al.* 1992, Pegtel 1999). So tetraploidy may have allowed *Cochlearia* to colonise coastal habitats from inland refugia, then moving northward along the coast. The uplands further north would have remained glaciated longer that the lowlands and coast, so the colonisation of the northern mountains from the coast is more likely than a mountain to coast colonisation.

Many Brassicaceae thrived in the open, disturbed habitats of the late glacial period (Hurka & Nueffer 1997). A similar pattern of polyploidy followed by diversification has been postulated for other Brassicaceae taxa *Draba* (Brochmann 1992) *Cardamine pratensis* (Franzke & Hurka 2000) and *Capsella* (Hurka & Nueffer 1997). *Cochlearia*, particularly the ancestral diploid *C. pyrenaica* subsp. *pyrenaica*, may have been widespread in the late glacial period at mid-altitudes in between the mountains and coast before being displaced by more competitive species. Once plants are adapted to the cold, they require high light intensities (Billings 1974). Plants in competitive environments need to tolerate low light conditions because the seedlings must germinate and grow while shaded by other plants. The wide ecological preferences of *Cochlearia* may be related to its role as an early post glacial coloniser. Many more niches are available for exploitation by early colonisers than late colonisers. Many plants suited to the late glacial conditions retreated to low competition habitats having been out-competed in other places (Godwin 1949), as appears the case with the diploid *C. pyrenaica* subsp. *pyrenaica* (Gill *et al.*1978).

6.2.2 Adaptation

Cochlearia inhabit a broad range of habitats: shingle and sand beaches, sand dunes, coastal grassland, estuarine mud, saltmarsh, brackish marsh, bird cliffs, snow beds, base-rich ledges and flushes and habitats with high heavy metal content: serpentine debris and old mine workings (Nagy & Procter (1997), Nordal & Laane (1990), pers. obs. 2003, 2004. As discussed in previous chapters, different habitats exert differential selective pressures on populations. In order to survive and compete, plant populations must be adequately adapted to the habitat in which they grow. The adaptations required to live in saltmarsh at the coast

are somewhat different to those required to live in base-rich flushes in the mountains. These habitats have very different characteristics e.g. the availability of fresh water, the NaCl concentration, the range of air temperatures, levels of disturbance and levels of competition.

Although genetic adaptation to different habitats cannot be detected directly with the data available, the persistence of some morphological forms in cultivation and not others indicates that a mixture of genetic adaptation and phenotypic plasticity has led to the occurrence eco-morphotypes in *Cochlearia*. (Nordal & Stabbetorp 1990, Pegtel 1999, pers. obs. 2004, 2005). Some of the morphological forms seem highly likely to be adaptations to specific habitats, for example the higher altitude populations have 'alpine' adaptations: small leaves, rosette form, a large tap root and some vegetative reproduction.

A study of three Brassica species showed that the polyploids had remarkably plastic genomes (Lukens *et al.* 2004). So, with the same genome, different populations can grow successfully in different habitats. This flexibility, rather than adaptation, was often used as the sole explanation for broader ecological tolerances of polyploids. At that time the polyploid genome was thought to have a lower capacity to fix adaptations than the diploid genome. However, evidence is increasing that polyploids also show faster rate of genomic change than diploids (Song *et al.* 1995) and therefore an increased capacity for quick genetic adaptation than diploids. Ecological flexibility that was previously attributed to phenotypic plasticity in polyploids may have been genetically based adaptation.

Gene flow appears quite low among many of the populations, which could increase rates of local adaptation, because non-locally adapted genotypes rarely arrive in populations. If ecotypes develop reproductive isolation mechanisms then they will start to diverge into separate lineages. The only possible example of this was found at Port Gheiraha where two different ecotypes living sympatrically were significantly genetically differentiated. However, the high genetic differentiation ($\Phi st = 0.26$) between two sympatric populations means that recent arrival of one of the populations from another site, is more likely than sympatric divergence.

6.3 Taxonomy

It was difficult to connect individuals and populations of *Cochlearia* with described taxa. The groupings of genetic and morphological variation did not correlate with taxonomic groupings. Local adaptations have created many *Cochlearia* morphotypes and ecotypes resulting in a confusing array of continuous variation. Within this array of variants poorly defined groupings can be made, which probably depend on similarities in the selective pressures on those groups. This kind of variation is the hallmark of a complex species and has led to taxonomic difficulties in other plant groups, e.g. *Cardamine pratensis* (Lihova *et al.* 2003), *Draba* (Scheen *et al.* 2002), *Minthostachys* (Schmidt-Lebuhn 2007) and *Eriastrum densifolium* (Brunell & Whitkus 1998).

6.3.1 The *Cochlearia officinalis* s.l. complex, one species or many?

Assignment of taxa to species rank commonly carries the implication of monophyly and reproductive isolation (Ereshefsky 2002). Although in reality most species are still defined morphologically rather than phylogenetically or using breeding studies, so neither of these assumptions may be true. Unlike the Biological Species Concept (BSC - Mayr 1942), the Phylogenetic Species Concept (PSC) allows for polyphyly and gene flow between species where it does not alter the independent trajectory of lineages (Nixon & Wheeler 1990). A definition of the phylogenetic species concept (PSC) as given by Nixon & Wheeler (1990) is as follows: 'the smallest aggregation of populations (sexual) or lineages (asexual) diagnosable by a unique combination of character states in comparable individuals'. If the PSC is applied to *Cochlearia officinalis* s.l. instead of the BSC, correlated characters differences are still required to define groups. This study has not been able to identify correlated character differences for the delimitation of *Cochlearia* taxa. In practice, clusters within continuous morphological variation are often given species epithets even in the absence of real character differences. Phenetic clusters in datasets, of the sort generated by PCA analysis of morphological or PCO analysis of AFLP data, can occur in the absence of character differences between those clusters (Goldstein et al. 2000). The less stringent the criteria used for classifying species, the more likely that the resultant species will not be independent, monophyletic lineages.

The practise of delimiting species is often much more complex than it would appear from generalised species concepts. An attempt to apply all feasible concepts and approaches to the *Cochlearia officinalis* s.l. complex in Britain would be extremely confusing. This section

will focus on comparing the variation in *Cochlearia* with the working criteria used by Hedrén (2004) to describe taxonomic approaches to another complex species *Carex flava*.

Hedrén (2004) proposed 6 levels of diversity (Table 6.1) that are each defined by a set of taxonomic criteria, level one being the most inclusive. The 'taxonomic species' (level 4) is the level at which most practising taxonomists would delimit species. To meet the criteria for a taxonomic species, taxa must be clearly separable by morphological characters, although they may be connected by low numbers of intermediate plants. Taxa within the *Cochlearia officinalis* complex do not meet these criteria as they cannot be clearly separated by morphological characters. The 'evolutionary species' (level 5) requires that taxa have evolved independently for a period of time and become separate lineages. These taxa may be on the boundary of a hierarchical and reticulate relationship, but they must be distinctive over most of their range. For *Cochlearia* taxa to fulfil these criteria they would have to form separate groupings of genetic variation, indicating lineages, which they do not. The variation within *Cochlearia* is between the 'evolutionary species' and the 'ecological species' (level 6 – occupation of minimally different adaptive zones). Only taxonomists with a very narrow species concept would define species at this level. The habitats in which *Cochlearia* occur could be described as more than minimally different, but the variation is based on ecological niche differences and local adaptation, rather than independent lineage formation.

Delimitation levels	Criteria for delimitation of taxa at each level.
Level 1: Biological Species I	Species should be characterised by different alleles at some loci and clear morphological character differences.
Zone Level 1 ↔ Level 2	Hybridisation, occasional introgression, infertile F1s
Level 2: Biological Species II	As Level 1, but morphological character differentiation may be less clear; quantitative trait differences still required.
Zone Level 2 ↔ Level 3	Hybridisation, but introgression infrequent
Level 3: Biological Species III	Distinct character states for qualitative morphological characters are sufficient. Taxa can be delimited where meiosis between hybrids is seriously disturbed.
Zone Level 3 ↔ Level 4	Moderate levels of hybridisation and introgression. F1s with reduced fertility.
Level 4: Taxonomic Species	Taxonomic species, morphological character separation. Low numbers of intermediate plants of reduced fertility are permitted under this definition.
Zone Level 4 ↔ Level 5	Hybridisation and introgression may occur, possibly minor reduction in fertility for F1s.
Level 5: Evolutionary Species	Evolutionary species, lineages mostly independent through time. Taxa remain genetically distinct over most of their distribution, but may be hard to separate morphologically.
Zone Level 5 ↔ Level 6	Poor morphological and genetic differentiation intermediates fertile.
Level 6: Ecological Species	Paraphyletic, with differentiation in habitat requirements.

Table 6.1 Summary of levels at which species have been delimited by different taxonomists in the *Carex flava* complex, adapted from Hedrén (2004). Level 1 is the most inclusive level of species delimitation, level 6 is the least inclusive. The level at which species are defined depends on the species concept of the taxonomist, although most practising taxonomists would probably define species around level 4 (Hedrén 2004).

6.3.2 Should intraspecific taxa be delimited?

If we consider all of the taxa within the *C. officinalis* s.l. complex (i.e. *C. officinalis* s.s., *C. atlantica*, *C. officinalis* subsp. *scotica*, *C. micacea*, *C. pyrenaica* subsp *pyrenaica* and *C. pyrenaica* subsp *alpina*) as one species, we must then consider whether or not to use intra-species categories to describe the variation. The *C. officinalis* s.l. complex contains a great deal of variation. If we look to taxonomic treatments for other similar groups, we see a range of different ways of treating polymorphic species that result from the removal of species epithets within complex groups. Lihová *et al.* (2002) argue in favour of 'lumping' species together without subspecies groups for *Cardamine pratensis* because delimitation is almost impossible and any names applied to the group do not describe the continuous nature of the variation. A similar approach is advocated for *Eriastrum densifolium*, the authors here state that intraspecific taxa can obscure information and suggest continuities or discontinuities where there are none (Brunell & Whitkus 1998). One drawback of 'lumping' is that

information about variation, ecotypes and morphotypes attached to species names may be lost. Schmidt-Lebuhn (2005) has argued against 'lumping' in the complex species *Minthostachys* because of the loss of information that it would entail. Clearly these cases may not be equivalent; indeed Mishler & Donoghue (1982) recommend that complex species are treated on a case by case basis, as no general recommendations can ever hope to apply in all situations.

The *Cochlearia officinalis* s.l. ecotypes of Scandinavia were dealt with by giving each a subspecies classification (Nordal & Laane 1990). If this approach was applied to British *Cochlearia*, the problem of distinguishing the subspecies would remain as intractable as the problem of distinguishing the species. Subspecies may be used when the variation they describe is not clear enough to warrant a species epithet; however this calls into question whether they represent anything at all. It has been noted that subspecies often contain discordant characters and cannot be objectively recognised (Wilson & Brown 1953, Stace 1986). A compromise is to recognise ecotypes, acknowledging that their boundaries cannot be clearly defined, without giving species or subspecies status, as has occurred in *Cardamine pratensis* (Lihova *et al.* 2003), *Liparis loeseii* (Pillion *et al.* 2007) and *Gentianella* (Winfield *et al.* 2003).

The term 'ecotype' has been used in this thesis to describe groups of populations of like-morpholology that inhabit similar habitats. An ecotype is not an official nomenclatural rank, and is normally used loosely to describe phenotypes of a species that are found in specific habitats, with no implication that they are genetically related. This is close to the definition paraphrased from Cain (1953) 'closely related, but ecologically distinct forms [that] either totally intergrade in nature where they meet, or appear completely interfertile under artificial conditions'. The International Code of Botanical Nomenclature gives five official categories to cover intraspecific variation: subspecies, variety, subvariety, forma and subforma (Grueter 1994). The ecotypes of *Cochlearia*, some which are currently described as species, sit between the official taxonomic terms of subspecies and forma. Subspecies being defined as: 'a geographically defined aggregate of local populations which differ taxonomically from other subdivisions of the species' (Mayr 1963). The unit of variation within the *C. officinalis* s.l. complex is the population and the individual, rather than a geographical grouping. The lack of regional similarities, means that a 'geographically defined aggregate of local populations', does not well describe the variation in the *Cochlearia officinalis* s.l. complex. 'Form' (or forma) is used much less frequently than subspecies and is defined as: 'distinctive

phenotypes of no persistent populational significance' (Cronquist 1988). 'Distinctive phenotypes' are certainly found within the *Cochlearia officinalis* s.l. complex. The locally adapted types of *Cochlearia* do seem to be persistent and specifically adapted, and so cannot be described as 'of no persistent populational significance'. If taxonomic delimitation was based on the ecotypes, then it is important to ensure that they themselves can be delimited. Ecotypes may vary continuously, just as species do (Linhart & Grant 1996). This kind of continuous variation appears to occur among *Cochlearia* ecotypes and additionally we know that the ecotypic groupings would not have any genetic basis. Therefore a classification based on ecotypes would encounter the same obstacles as any other classification system.

6.3.3 Specific discussion of the taxa within the *Cochlearia officinalis* s.l. complex

6.3.3.1 *C. officinalis* s.s.

This taxon contains a very distinctive large form that grows in response to high nutrient levels e.g. at bird cliffs sites, but is maintained when cultivated in standard conditions (as shown in Figure 3.3, Chapter 3). These plants are much larger and more robust than all other *Cochlearia* ecotypes, and are the most visually distinctive part of the complex. However, even this most distinctive form, does not show as a genetic similarity cluster in AFLP marker analysis. The morphological data show that the distinction is size-based, rather than shape-based, and that there is a great deal of variation between populations and individuals within the taxon. Other ecotypes classified under *C. officinalis* s.s. are often hard to tell apart from *C. officinalis* subsp. *scotica* and *C. atlantica*.

6.3.3.2 *C. officinalis* subsp. *scotica*

Cochlearia officinalis subsp. *scotica* does not seem to be a meaningful group within the Scottish coastal *Cochlearia*, but rather an arbitrary part of a highly variable species. *Cochlearia officinalis* subsp. *scotica* was formerly *C. scotica*, but the taxon was changed to a subspecies of *C. officinalis* s.s. due to doubts over its distinctiveness. It may be similar to the small form found in Scandinavia on low nutrient sites which does not increase in size with the addition of nutrients (Nordal *et al.* 1986). The results of this genetic study show that *C. officinalis* subsp. *scotica* type plants are part of an assemblage of interconnected populations around the coast.

6.3.3.3 *C. atlantica*

This taxon has a poorly defined niche and is difficult to distinguish from *C. officinalis* s.s. and *C. officinalis* subsp. *scotica* types. It was delimited based largely on herbarium specimens by Pobedimova (1970, 1971). Herbarium specimens may be preferentially collected either because they are unusual or because they are good examples of a taxon, creating a collection that emphasises extreme forms. The continuous nature of variation in the Scottish coastal *Cochlearia* may not have been apparent in a herbarium-based study. The plants tend to have truncate-based leaves, but are morphologically no more or less distinctive than the other taxa described within the *C. officinalis* s.l. complex. The *C. atlantica* plants from the type location were not strongly differentiated from upland plants on Ben Nevis nearby (Fst = 0.16) and the populations clustered next to each other on PCO analysis. Therefore in line with the general conclusions, *C. atlantica* appears to be a morphological type that appears in response to certain habitats.

6.3.3.4 *C. pyrenaica* subsp. *pyrenaica*

The samples of this taxon that were included in the analysis were not genetically distinctive from the other upland species; however this taxon was not sampled thoroughly enough to draw firm conclusions. As the ancestral diploid, the difference in chromosome number between *C. pyrenaica* subsp. *pyrenaica* and the other tetraploid taxa may create undiagnosed reproductive barriers. Crossing experiments were carried out between *C. pyrenaica* and *C. officinalis* s.s., although the subspecies was not specified, there was some loss of fertility in F1 hybrids of these crosses (Gill 1973 – shown in Table 3, Chapter 1).

6.3.3.5 *C. pyrenaica* subsp. *alpina*

No major differentiation was found between the tetraploid inland populations of *C. pyrenaica* subsp. *alpina*. The putative *C. pyrenaica* subsp. *alpina* cultivated in standard conditions (Chapter 4) produced much more variable morphologies compared with the plants in the wild populations. This highlights the influence of environmental variables on upland *Cochlearia*. In turn the distinction between upland and coastal taxa was revealed as artificial. There is no evidence that the inland tetraploid form lineages separate from the coastal tetraploid species.

6.3.3.6 *C. micacea*

No evidence was found for a distinct genetic or morphological grouping that could be referred to *C. micacea*. Even at the type location, the two populations showed a mixture of

morphological characters, some of which fitted the description of *C. micacea*, some of which were closer to the description of *C. pyrenaica* subsp. *alpina.* The existence of the putative endemic *C. micacea* is doubtful, at most, some of the populations in the uplands appear to be a form adapted to higher altitude montane conditions. It appears that extreme morphological forms were described as *C. micacea*, but that these are part of an almost continuously variable assemblage that has responded to differing ecological conditions.

6.3.4 Taxonomic summary

The *Cochlearia officinalis* complex does not contain separate evolutionary lineages, and so the species cannot be represented as end nodes in a hierarchal phylogenetic scheme (Goldstein et al. 2000). Some species concepts may accommodate these polyphyletic groups as species, but the variation within the complex does not even meet the least stringent criteria for delimitation - phenetic clustering of morphological or genetic characters. Complex groups delimited as a single species can be more variable than a species in a stable evolutionary phase. Taking into consideration a history of taxonomic controversy, along with the data presented in this thesis, it appears that the *Cochlearia officinalis* s.l. complex cannot be divided confidently into either species or subspecies classifications and instead appears to consist of a series of ecotypic variants. This supports the use of a broad species concept that encompasses all the taxa currently delimited within it.

6.4 Conservation of biodiversity

The basic aim of conservation is to protect biodiversity (Moritz 2002). Conservation works with dynamic living systems, so it is not sufficient to think only in terms of contemporary biodiversity, but it is also it is necessary to plan and manage for future biodiversity (Moritz 2002, Cowling & Pressey 2001, Ennos *et al.* 2005). Plant populations face continuous change in environmental conditions and in order to survive they must tolerate changes or have the genetic capacity to adapt to them. Conservation of current diversity may not be adequate to ensure their future survival. In addition, a plant group which appears as one lineage at present may evolve to form a diverse group in the future. To protect future biodiversity the emphasis must be on maintaining the potential for adaptation and diversification. While the importance of genetic resources for future adaptation have been acknowledged and discussed by many workers in the field, the message has not filtered down to real changes in conservation policy. Even for taxonomically complex species, the conservation provision in Europe is still restricted to species action plans. Safeguarding the

processes in diversifying groups cannot be adequately achieved using existing policies (French 2003, Ennos *et al.* 2005).

6.4.1 Specific difficulties applying conservation to *Cochlearia officinalis* s.l.

An alternative approach for complex groups is the process-based approach (Moritz 2002, Ennos *et al.* 2005). Protecting diversifying processes for future biodiversity is challenging because the processes promoting biodiversity are uncertain in many groups. However, some generic steps that can be taken include: 1) Conservation of habitat areas where there is a high concentration of current diversity, with the suggestion that these habitats will continue to promote diversity in future. 2) Ensuring connectivity of habitats for progenitor species and high diversity areas, so that progenitors are supplied to the system that creates recurrently formed taxa. This may also promote gene flow between partially diverged lineages. 3) To maintain a range of diverse habitats that place differential selective pressures of plant populations and drive adaptive change. 4) To prioritise the protection of progenitor taxa over locally arisen variants or taxa that could easily be recreated (Moritz 2002, French 2003).

A limited number of recommendations for conservation within the *Cochlearia officinalis* s.l. complex can be made based on this policy. The diploid *C. pyrenaica* subsp. *pyrenaica* would have a higher priority than the tetraploid, because tetraploids could be recreated by another autopolyploid event, but the original diploid cannot be recreated again from the tetraploids. *Cochlearia* ecotypes currently named in species action plans *C. micacea* and *C. officinalis* subsp. *scotica* would have lower conservation status, because these rarer ecotypes are not evolutionarily separated from the widespread ecotypes. If a rare ecotype were lost, similar ecotypes could be re-created by adaptations of immigrant plants from other habitats (providing that similar habitat remained).

In order to adhere to this policy further it is necessary to clarify the origins and formation of diversity and patterns variation in the target group. Then populations must be prioritised in order to protect the processes that sustain current and future diversity (Moritz 2002). A more comprehensive process-based plan was created by French (2003) for the conservation of the complex species *Euphrasia* in Britain. Unfortunately, the characteristics of variation in the *C. officinalis* s.l. complex, namely: the low level of structure and variation in the AFLP data; the lack of clear morphological characters and low chloroplast variation, mean that it has not been possible to gather enough information for a detailed process-based plan for *C. officinalis* s.l. We cannot choose populations or ecotypes that are most likely to maintain

diversification processes given the current uncertainty as to what drives the diversification process in the British *C. officinalis* s.l. complex. Specific conservation recommendations for the *C. officinalis* s.l complex cannot be made beyond the abandonment of the species action plans for *C. micacea* and *C. officinalis* subsp *scotica* (named in the species action plan as *C. scotica*). Taxonomic controversy has stalled any conservation efforts for these two taxa, so an abandonment of species action plans for the genus does not signify a real change in management or a greater risk of loss of diversity.

6.4.2 Conservation recommendations

The conservation or biodiversity value of the *C. officinalis* s.l. complex is unclear. The variation between named taxa within the *C. officinalis* s.l. complex is at a level that most people would consider within-species variation. Unquantified adaptive variation and phenotypic plasticity seem to be at the root of the remarkable ecological variation in *C. officinalis* s.l. complex in Britain. The complex may warrant protection as a future source of diversity. However, because of the level of uncertainty, specific conservation efforts for the complex are not recommended.

An alternative habitat based approach can be recommended that uses the information available about the ecotypes and distribution of *Cochlearia*. Cowling & Pressey (2001) worked with diversifying taxa in the Cape Floristic Region (CFR) in South Africa. In the CFR, there are many closely related taxa that have undergone diversification driven by ecological niche adaptation. There are large clusters of closely related species, whose relationships cannot be resolved phylogenetically. Cowling & Pressey (2001) recommend an over-arching policy of habitat conservation to protect diversification processes of the ecosystem as a whole. South Africa is a floristically rich region, whereas Britain has low species diversity. However, diversifying groups in Britain are an important part of our resident biodiversity; therefore lessons can be learned from the CFR and applied here. Diverse *C. officinalis* s.l. habitats can be protected without exact knowledge of the diversification processes that are occurring. This approach is not targeted at conserving specific processes, but it has the advantage of benefiting biodiversity as a whole.

6.4.3 Current conservation status of *Cochlearia officinalis* s.l. and specific recommendations.

Cochlearia as a genus is widespread in Britain and does not face any major threats, apart from climate change (this issue is beyond the scope of this thesis). Grazing by deer and

sheep could be considered a threat to some coastal populations; however it may also promote local dwarf forms, thus driving diversification (Díaz *et al.* 2007). Some specific forms and ecotypes are more infrequent, these include *C. micacea* and *C. officinalis* subsp. *scotica*. There are other equally unusual morphological forms that have not been named or given conservation status, e.g. the shingle dwelling *Cochlearia* and the metalliferous *Cochlearia*.

The distribution of *Cochlearia officinalis* s.l. is fragmented in the uplands, but this distribution pattern is determined by *Cochlearia officinalis* s.l. habitat preferences, i.e. either base rich sites with constant flow of water or serpentine gravel. The coastal habitats of *Cochlearia* in Scotland are similarly separated by areas of unsuitable habitat, rather than by anthropogenic fragmentation. There are many *Cochlearia officinalis* s.l. populations in remote areas with low human impact. Many coastal *Cochlearia officinalis* s.l. populations studied are already afforded indirect protection because they grow in priority habitats, under the Biodiversity Habitat Action plans (e.g. coastal and floodplain grazing marsh, coastal saltmarsh, coastal vegetated shingle and maritime cliff and slopes). Upland *Cochlearia* grow on pockets of base-rich rock in the generally acidic species-poor highlands. An adequate proportion of the sites are SSSIs or nature reserves e.g. Ben Lui National Nature Reserve (NNR) and Ben Lawers NNR. In no case has this protection been afforded solely because of the presence of priority species *C. micacea* or *C. officinalis* subsp *scotica*. The level of threats to *C. micacea* populations was judged to be low in the 1994 survey (Dalby & Rich 1994). The continued general protection of these habitats should be sufficient to prevent threats to adaptive forms of *Cochlearia*.

6.5 Further research

This thesis concentrated on the putative endemic species of Scotland. A small scale morphological and genetic marker study to clarify the relationship between *C. anglica*, *C. danica* and the *C. officinalis* complex may be necessary. These taxa are easily distinguished from the taxa within the *C. officinalis* s.l. complex. However, they can hybridise with taxa in the *C. officinalis* s.l. complex. AFLP markers may yield useful information at hybrid zones between the *C. officinalis* s.l. complex and *C. anglica* or *C. danica*. The lack of chloroplast variation means that colonisation routes and population history cannot be deduced. Further mapping of the distribution of chromosome counts in the UK would help to define the distribution of the ancestral diploid *C. pyrenaica* subsp. *pyrenaica.* Further study to define the relationship between the diploid ancestral populations and the tetraploid populations may

give more information about the processes that occurred during polyploidisation and colonisation.

The lack of taxonomic or geographical signal in the *Cochlearia officinalis* s.l. complex from AFLP data indicates that further investigation of general neutral genetic variation in *Cochlearia* in Britain would not be worthwhile. A study of the adaptive mechanisms in *Cochlearia* would be the best way to investigate how complex variation has arisen in the group. This could be achieved very satisfactorily in a low-tech manner, with reciprocal transplant and common garden experiments. The response of *Cochlearia* to environmental variables could also be investigated by searching for variation at quantitative trait loci (QTL). The evolutionary proximity of *Cochlearia* to the very heavily investigated genus *Arabidopsis* means that it could be possible to link adaptive mechanisms directly to genes with known functions.

Further genetic studies in the genus *Cochlearia* on a Europe-wide scale, or better across its circumpolar distribution, are more likely to be fruitful than further genetic studies confined to the British Isles. Previous taxonomic studies at a Europe-wide geographical scale have revealed useful information (Koch *et al.* 1996, Koch *et al.* 1998). One of the major limitations of this study and of some other previous studies in *Cochlearia* is that they have a narrow geographical scope. The taxonomic treatments which seem appropriate in Britain may not be applicable in other regions. A global standardisation of taxonomic treatments in *Cochlearia* and in other similar groups should be the ultimate aim of further taxonomic investigations.

7. References

Abbott RJ, Brochmann C (2003) History and evolution of the arctic flora: in the footsteps of Eric Hultén. Molecular Ecology, 12, 299-313.

Abbott RJ, Chapman HM, Crawford RMM, Forbes DG (1995) Molecular diversity and derivations of populations of *Silene acaulis* and *Saxifraga oppositifolia* from the high Arctic and more southerly latitudes. Molecular Ecology, 4, 199-207.

Abs C (1999) Differences in the life history of two *Cochlearia* species. In: Ecology of closely related plant species (eds. Marhold K, Schmid B and Krahulec F), pp. 33-45. Opulus Press, Uppsala.

Agrawal AA (2001) Phenotypic Plasticity in the Interactions and Evolution of Species Science, 294, 321-326.

Allaby RG, Brown TA (2003) AFLP data and the origins of domestic crops. Genome, 46, 448-453.

Al-Shehbaz A, Beilstein MA, Kellogg EA (2006) Systematics and phylogeny the Brassicaceae (Cruciferae): an overview. Plant Systematics and Evolution, 259, 89-120.

Amos W, Harwood J (1998) Factors affecting levels of genetic diversity in natural populations. Philosophical Transactions of the Royal Society of London, Series B Biological Sciences, 353, 177-186.

Angiolillo A, Mencuccini M, Baldoni L (1999) Olive genetic diversity assessed using amplified fragment length polymorphisms. Theoretical and Applied Genetics, 98, 432-2242.

Ashton PA, Abbott RJ (1992) Multiple origins and genetic diversity in the newly arisen allopolyploidy species *Senecio cambrensis* Rosser. (Compositeae). Heredity, 68, 25-32.

Babington CC (1843) Manual of British Botany. John Van Voorst, London.

Baker AJM, Dalby DH (1980) Morphological variation between some isolated populations of *Silene maritima* With. in the British Isles with particular reference to inland populations of metalliferous soils. New Phytologist, 84, 123-138.

Bauert M (1996) Genetic diversity and ecotypic differentiation in Arctic and Alpine populations of *Polygonium viviparum*. Arctic and Alpine research, 28, 190-195.

Beeby WH (1889) On the Flora of Shetland. The Scottish Naturalist, 9, 20-32.

Beeby WH, (1898) *Cochlearia officinalis* L. x *micacea* Marshall. Botanical Exchange Society, 1, 510-511.

Billings WD (1974) Adaptations and origins of alpine plants. Arctic and Alpine research, 6, 129-142.

Blanc G, Wolfe KH (2004) Functional divergence of duplicated genes formed by polyploidy during *Arabidopsis* evolution. The Plant Cell, 16, 1679-1691.

Bortiri E, Jiang S-HOJ, Baggett S, Granger A, Weeks C, Buckingham M, Potter D, Parfitt DE (2001) Phylogeny and systematics of *Prunus* (Rosaceae) as determined by sequence analysis of ITS and the chloroplast trnL-trnF spacer DNA. Systematic Botany, 26, 797-807.

Breyne P, Rombaut D, Van Gysel A, Van Montagu M, Gerats T (1999) AFLP analysis of genetic diversity within and between *Arabidopsis thaliana* ecotypes. Molecular and General Genetics, 261, 1432-1874.

Brochmann C (1992) Polyploid evolution in arctic alpine *Draba* (Brassicaceae). Sommerfeltia supplement, 4, 1-37.

Brochmann C, Brysting AK, Alsos IG, Borgen L, Grundt HH, Scheen A-C, Elven R (2004) Polyploidy in Arctic Plants. Biological Journal of the Linnean Society, 82, 521-536.

Brochmann C, Elven R (1992) Ecological and genetic consequences of polyploidy in arctic *Draba* (Brassicaceae). Evolutionary Trends in Plants, 6, 111-123.

Brochmann C, Gabrielsen T, Nordal I, Landvik JY, Elven R (2003) Glacial survival or tabula rasa? The history of the North Atlantic biota revisited. Taxon, 52, 417-450.

Brochmann C, Stedje B, Borgen L (1992) Gene flow across ploidal levels in *Draba* (Brassicaceae). Evolutionary Trends in Plants, 6, 125-134.

Bronken P, Taberlet P, Gielly L, Brochmann C (2001) Chloroplast and nuclear DNA variation on a circumpolar scale: migration history of the clonal *Saxifraga cernua.* Bauhinia, 15, 75.

Brunell MS, Whitkus R (1999) Assessment of morphological variation in *Eriastrum densifolium* (Polemoniaceae): Implications for subspecific delimitation and conservation. Systematic Botany, 23, 351-368.

Bussell (1999) The distribution of random amplified polymorphic DNA (RAPD) diversity amongst populations of *Isotoma petraea* (Lobeliaceae). Molecular Ecology, 8, 775-789.

Cain AJ (1952) Geography, ecology and coexistence in relation to the biological definition of the species. Evolution, 7, 76-83.

Campbell D, Bernatchez L (2004) Generic scan using AFLP markers as a means to assess the role of directional selection in the divergence of sympatric whitefish ecotypes. Molecular Biology and Evolution, 21, 945-956.

Chay P, Thurling N (1989) Variation in pod length in spring rape (*Brassica napus*) and its effect on seed yield components. Journal of agricultural science, 113, 139-147.

Chung M, Gelembiuk G, Givnish TJ (2004) Population genetics and phylogeography of endangered *Oxytropis campestris* var. *chartacea* and relatives: arctic-alpine disjuncts in Eastern North America. Molecular Ecology, 13, 3657-3673.

Cieślak E, Ronikier M, Koch MA (2007) Western Ukrainian *Cochlearia* (Brassicaceae) - the identity of an isolated edge population. Taxon, 56, 112-118.

Clapham AR (1952) *Cochlearia*. In: Flora of the British Isles (eds. Clapham AR, Tutin TG and Warburg EF), pp. 188-192. Cambridge University Press, Cambridge.

Clapham AR (1981) *Cochlearia*. In: Excursion Flora of the British Isles (eds. Clapham AR, Tutin TG and Warburg EF), pp. 56-59. Cambridge University Press, Cambridge.

Clapham AR, Tutin TG, Moore DM (1987) Flora of the British Isles. Cambridge University Press, Cambridge.

Clarke S (1993) A survey of *Cochlearia micacea* on Ben Lawers NNR RF23.09. A report for Scottish Natural Heritage.

Comes HP, Kadereit JW (1998) The effect of Quaternary climatic changes on plant distribution and evolution. Trends in Plant Science, 11, 432-438.

Comes HP, Kadereit JW (2003) Spatial and temporal patterns in the evolution of the flora of the European Alpine System. Taxon 52, 451-462.

Cowling RM, Pressey RL (2001) Rapid plant diversification: Planning for an evolutionary future. Proceedings of the National Academy of Sciences of the USA, 98, 5452-5457.

Cronquist A (1988) The evolution and classification of flowering plants. 2nd Edition. Columbia University Press, New York.

Dalby DH (1990) A new combination in *Cochlearia* (Cruciferae) Watsonia, 18, 82.

Dalby DH (1991) *Cochlearia*. In: Crucifers of Great Britain and Ireland (ed. Rich TGC), pp. 262-275. BSBI Handbook Number 6. Botanical Society of the British Isles, London.

Dalby DH, Rich TCG (1994) The history, taxonomy, distribution and ecology of mountain scurvy grass (*Cochlearia micacea* Marshall). A report for Scottish Natural Heritage, Project report number 42.

Dauvergne X, Cérantola S, Magné C, Deslandes E, Bessières MA (2006) Evidence for the common occurrence of the glucosinolate glucoputranjivin in the genus *Cochlearia*. Biochemical Systematics and Ecology, 34, 596-598.

Després L, Gielly L, Redoutet B, Taberlet P (2003) Using AFLP to resolve phylogenetic relationships in a morphologically diversified plant species complex when nuclear and chloroplast sequences fail to reveal variability. Molecular Phylogenetics and Evolution, 30, 158-196.

Després L, Loriot S, Gaudeul M (2002) Geographic pattern of genetic variation in the European globeflower *Trollius europaeus* L. (Ranunculaceae) inferred from amplified fragment length polymorphism markers. Molecular Ecology, 11, 2337-2347.

Díaz S, Lavorel S, Mcintyre S, Falczuk V, Casanoves F, Milchunas DG, Skarpe C, Rusch G, Sternberg M, Noy-Meir I, Landsberg J, Wei Z, Clark H, Campbell BD (2007) Plant trait responses to grazing – a global synthesis. Global Change Biology, 13, 313-341.

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf material. Phytochemical Bulletin, 19, 11-15.

Druce GC (1929) Notes on the second edition of the British Plant list. Report of the Botanical Exchange Club of the British Isles, 8, 867-883.

Efeshefsky M (2002) Linnaean ranks: vestiges of a bygone era. Philosophy of Science, 69, S305-S315.

Ehrich D, Gaudeul M, Assefa A, Koch MA, Mummunhoff K, Nemomissa S, Intrabiodiversity consortium, Brochmann C (2007) Genetic consequences of Pleistocene range shifts: contrast between the Arctic, the Alps and the East African mountains. Molecular Ecology, 16, 2542-2559.

Elkington TT (1984). Cytogenetic variation in the British Flora: origin and significance. New Phytologist, 98, 101-118.

Ennos RA, Cowie CJ, Legg C, Sydes C (1997) Which measures of genetic variation are relevant in plant conservation? A case study of *Primula scotica*. In: The role of genetics in conserving small populations (eds. Tew TE, Crawford TJ, Spencer JW, Stevens DP, Usher MB and Warren J), pp. 73-79. Joint Nature Conservancy Council (JNCC), Peterborough, UK.

Ennos RA, French GC, Hollingsworth PM (2005) Conserving taxonomic complexity. Trends in Ecology and Evolution, 20, 164-168.

Ennos, RA, Sinclair, WT, Hu, X-S, Langdon, A (1999) Using organelle markers to elucidate the history, ecology and evolution of plant populations. In: Molecular Systematics and Plant Evolution (eds. Hollingsworth PM, Bateman RM, Gornall RJ), pp. 1-19. Taylor & Francis, London.

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics, 131, 479-491.

Ferne GM (1977) A morphological and cytological investigation of *Cochlearia* on the Gower Peninsula, Glamorgan. New Phytologist, 79, 455-458.

Fjellheim S, Elven R, Brochmann C (2001) Molecules and morphology in concert. II. The *Festuca brachyphylla* complex (Poaceae) in Svalbard. American Journal of Botany, 88, 869-882.

Forrest AD, Hollingsworth ML, Hollingsworth PM, Sydes C, Bateman RM (2004) Population genetic structure in European populations of *Spiranthes romanzoffiana* set in the context of other genetic studies on orchids. Heredity, 92, 218-227.

Franzke A, Hurka H (2000) Molecular systematics and biogeography of the *Cardamine pratensis* complex (Brassicaceae). Plant systematics and Evolution, 224, 213-234.

Franzke A, Pollman K, Bleeker W, Kohrt R, Hurka H (1998) Molecular systematics of *Cardamine* and allied genera (Brassicaceae): ITS and non coding chloroplast DNA. Folia Geobotanica, 33, 225-240.

French GC (2003) Conservation genetics of the critical plant genus *Euphrasia* L. in Britain. PhD thesis, The University of Edinburgh.

Galen C, Shore JS, Deyoe H (1991) Ecotypic divergence in alpine *Polemonium viscosium*: genetic structure, quantitative variation, and local adaptation. Evolution, 45, 1218-1228.

Gaudeul M, Taberlet P, Till-Bottraud I (2000) Genetic diversity in an endangered alpine plant, *Eryngium alpinum* L. (Apiaceae), inferred from amplified fragment length polymorphism markers. Molecular Ecology, 9, 1625-1637.

Gill, JJB (1965). Diploids in the Genus *Cochlearia*. Watsonia, 6, 188-189.

Gill JJB (1971a) *Cochlearia scotica* Druce-does it exist in Northern Scotland? Watsonia, 8, 395-402.

Gill JJB (1971b) The cytology and transmission of accessory chromosomes in *Cochlearia pyrenaica* DC. (Cruciferae). Caryologica, 24, 173-181.

Gill JJB (1971c) Cytogenetic studies in *Cochlearia* L: The chromosomal homogeneity within both the 2n =12 diploids and the 2n = 14 diploids and the cytogenetic relationship between the two chromosome levels. Annals of Botany, 35, 947-956.

Gill JJB (1973) Cytogenetic studies in *Cochlearia* L. (Crucifereae). The origins of *C. officinalis* L. and *C. micacea* Marshall. Genetica 44, 217-234

Gill JJB (1976). Cytogenetic studies in *Cochlearia* L. (Crucifereae): the chromosomal constitution of *C. danica* L. Genetica, 46, 115-127.

Gill JJB, McAllister HA, Fearn GM (1978) Cytotaxonomic studies on the *Cochlearia officinalis* L. group from inland stations in Britain. Watsonia, 12, 15-21.

Godwin H (1964) Late-Weichselian conditions in South-Eastern England: organic deposits at Colney Heath, Herts. Proceedings of the Royal Society of London. Series B, Biological Sciences, 160, 258-275.

Goldstein PZ, Desalle R, Amato G, Vogler AP (2000) Conservation Genetics at the Species Boundary. Conservation Biology, 14, 120-131.

Gregor JW (1938) Experimental Taxonomy II. Initial Population differentiation in *Plantago maritima* L. of Britain. New Phytologist, 37, 15-49.

Grueter W, McNeill J, Barrie FR (1994) Report on botanical nomenclature – Yokahama 1993. Englera, 14, 198-205.

Griffith C, Kim E, Donohue K (2004) Life-history variation and adaptation in the historically mobile plant *Arabidopsis thaliana* (Brassicaceae) in North America. American Journal of Botany, 91, 837-849.

Group UB (1995) Biodiversity: the UK steering group report. HMSO, London.

Grundt HH, Popp M, Brochmann C, Oxelman B (2004) Polyploid origins in a circumpolar complex in *Draba* (Brassicaceae) inferred from cloned nuclear DNA sequences and fingerprints. Molecular Phylogenetics and Evolution, 32, 695-710.

Guggisberg A, Mansion G, Kelso S, Conti E (2006) Evolution of biogeographic patterns, ploidy levels and breeding systems in a diploid-polyploid species complex of *Primula*. New Phytologist, 171, 617-632.

Guo YP, Saukel J, Mittermayr R, Ehrendorfer F (2005) AFLP analyses demonstrate genetic divergence, hybridisation, and multiple polyploidisation in the evolution of *Achillea* (Asteraceae-Anthemideae). New Phytologist, 166, 273-290.

Hamrick JL, Godt MJW (1990) Allozyme diversity in plant species. In: Plant population genetics, breeding and genetic resources (eds. Brown AHD, Clegg MT, Kahler AL, Weir BR), pp. 43-63. Sinauer Associates Inc, Sunderland, Massachusetts.

Hedrén M (2004) Species delimitation and the partitioning of genetic diversity – an example from the *Carex flava* complex (Cyperaceae). Biodiversity and Conservation, 13, 293-316.

Hedrén M, Fay MF, Chase MW (2001) Amplified Fragment length polymorphisms (AFLP) reveal details of polyploid evolution in *Dactylorhiza* (Orchidaceae). American Journal of Botany, 88, 1868-1880.

Hewitt GM (2004) Genetic consequences of climatic oscillations in the Quarternary. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences, 359, 183-195.

Henderson A (2006) Traditional morphometrics in plant systematics and its role in Palm systematics. Botanical Journal of the Linnean Society, 151, 103-111.

Hillis DM (1987) Molecular versus morphological approaches to Systematics. Annual Review of Ecology and Systematics, 18, 23-42.

Holderegger R, Abbott RJ (2003) Phylogeography of the Arctic-Alpine *Saxifraga oppositifolia* (Saxifragaceae) and some related taxa based on cpDNA and ITS sequence variation. American Journal of Botany, 90, 931-936.

Hollingsworth PM (2003) Taxonomic complexity, population genetics, and plant conservation in Scotland. Botanical Journal of Scotland, 55, 55-63.

Hollingsworth PM, Ennos RA (2004) Neighbour joining trees, dominant markers and population genetic structure. Heredity, 94, 490-498.

Howe HF, Smallwood J (1982) Ecology of seed dispersal. Annual Review of ecology and systematics, 13, 201-228.

Hultén E (1970) The circumpolar plants II. Kungliga svenska Vetenskapsakademiens Handlingar 4 Serien, 13, 1-463.

Hurka H, Neuffer B (1997) Evolutionary processes in the genus *Capsella* (Brassicaceae). Plant Systematics and Evolution, 206, 295-316.

Ipek M, Simon P (2003) Sequence characterisation of polymorphic AFLP fragments in garlic (*Allium sativum* L.). Plant and Animal genome, Final Abstracts Guide, San Diego, California. Poster 685, pp. 245.

Jalas J, Suominen J, Lampinen R (Eds.) (1996) Atlas Flora Europaeae, 11 Cruciferae, Helsinki University printing house, Helsinki.

Jiménez-Ambriz G, Petit C, Bourrié I, Dubois S, Olivieri I, Ronce O (2007) Life history variation in the heavy metal tolerant plant *Thalaspi caerulecens* growing in a network of

contaminated and non contaminated sites in Southern France: role of gene flow, selection and phenotypic plasticity. New Phytologist, 173, 199-215.

Jones CJ, Edwards KJ, Castaglione S, Winfield MO, Sala F, van deWiel C, Bredemeijer G, Vosman D, Matthes M, Daly A, Brettschneider R, Bettini P, Buiatti M, Maestri E, Malcevshi A, Marmiroli N, Aert R, Volchaert G, Rueda J, Linacerro R, Vazquez A, Karp A (1997) Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. Molecular Breeding, 3, 381-390.

Jørgensen, S, Mauricio R (2004) Neutral genetic variation among wild North American populations of the weedy plant *Arabidopsis thaliana* is not geographically structured. Molecular Ecology, 13, 3403-3413.

Kardolus JP, van Eck HJ, van de Berg RG (1996) The potential of AFLPs in biosystematics: A first application in *Solanum* taxonomy (Solanaceae). Plant Systematics and Evolution, 210, 87-103.

Kik C, Van Andel J, Joenje W (1990) Life history variation in ecologically contrasting populations of *Agrostis stolonifera*. Journal of Ecology, 78, 962-973.

Koch M (2002) Genetic differentiation and speciation in pre-alpine *Cochlearia* Allohexaploid *Cochlearia barvarica* Vogt (Brassicaceae) compared to its diploid ancestor *Cochlearia pyrenaica* DC. in Germany and Austria. Plant Systematics and Evolution, 232, 35-49.

Koch M, Dobes C, Bernhardt KG, Kochjarová J (2003) *Cochlearia macrorrhiza* (Brassicaceae): A bridging species between *Cochlearia* taxa from the Eastern Alps and the Carpathians? Plant Systematics and Evolution, 242, 137-147.

Koch M, Hurka H, Mummenhoff K (1996) Chloroplast DNA restriction site variation and RAPD-analysis in *Cochlearia* (Brassicaceae) biosystematics and evolution. Nordic Journal of Botany, 16, 585-603.

Koch M, Huthmann M, Hurka H, (1998). Isozymes, Speciation and Evolution in the Polyploid Complex *Cochlearia* L. (Brassicaceae). Botanica Acta, 111, 411-425.

Koch MK, Mummenhoff K, Hurka H (1999). Molecular phylogenetics of *Cochlearia* (Brassicaceae) and allied genera based on nuclear ribosomal ITS DNA sequence analysis contradict traditional concepts of their evolutionary relationship. Plant Systematics and Evolution, 216, 207-230.

Kochjarová J, Valachovic M, Bures P, Mráz P (2006), The genus *Cochlearia* L. (Brassicaceae) in the Eastern Carpathians and adjacent area. Botanical Journal of the Linnean Society, 151, 355-364.

Kolseth A-K, Lönn M, (2005) Genetic structure of *Euphrasia stricta* on the Baltic Island of Gotland, Sweden. Ecography, 28, 443-452.

Kraft T, Nybom H (1995) DNA fingerprinting and biometry can solve some taxonomic problems in apomictic blackberries (*Rubus* subgen. *Rubus*). Watsonia, 20, 329-343.

Lambracht E, Westberg E, Kadereit J W (2007) Phylogeographic evidence for the postglacial colonisation of the north and Baltic sea coasts from inland glacial refugia by *Triglochin maritima* L. Flora, 202, 79-88.

Lang G (1995) Quartäre Vegetationsgeschichte Europas. Fischer, Jena, Stuttgart, New York.

Lefébvre C (1974) Population variation and taxonomy in *Armeria maritima* with a special reference to heavy metal tolerant populations. New Phytologist, 73, 209-219.

Levin DA (2001) The recurrent origin of plant races and species. Systematic Botany, 26, 197-204.

Lexer C, Kremer A, Petit RJ (2006) Shared alleles in sympatric oaks: recurrent gene flow is a more parsimonious explanation than ancestral polymorphism. Molecular Ecology, 15, 2007-2012

Lihová J, Tribsch A, Marhold K (2003) The *Cardamine pratensis* (Brassicaceae) group in the Iberian Peninsula: taxonomy, polyploidy and distribution. Taxon, 52, 783-802.

Lihová J, Marhold K, Kudoh H, Koch MA (2006) Worldwide phylogeny and biogeography of *Cardamine flexuosa* (Brassicaceae) and its relatives. American Journal of Botany, 93, 1206-1221.

Lin JJ, Kuo J (1995) AFLP, a novel PCR-based assay for plant and bacterial DNA fingerprinting. Focus, 17, 66-70.

Linhart YB, Grant MC (1996) Evolutionary significance of local genetic differentiation in plants. Annual Review of Ecology and Systematics, 27, 237-277.

Linnaeus C (1753) Species Plantarum, A facsimile of the first edition, 1, Ray Society, London.

Lukens LN, Quijada PA, Udall J, Pires JC, Schranz ME, Osborn TC (2004) Genome redundancy and plasticity within ancient and recent *Brassica* crop species. Biological Journal of the Linnean Society, 82, 665-674.

Macnair, M.R. (1989) The potential for rapid speciation in plants. Genome, 31, 203-210.

Magri DGG, Vendramin B, Comps B, Dupanloup I, Geburek T, Gomory D, Latalowa M, Litt T, Paule L, Roure JM, Tantau I, van der Knapp WO, Petit RJ, de Beaulieu J-L (2006) A new scenario for the Quarternaty history of European Beech populations: palaeobotanical evidence and genetic consequences. New Phytologist, 171, 199-221.

Mantel N (1967) The detection of disease clustering and a generalised regression approach. Cancer Research, 27, 209-220.

Marhold K (1996) Multivariate morphometric study of the *Cardamine pratensis* group (Cruciferae). Plant Systematics and Evolution, 200, 141-159.

Marhold K, Lihova J, Perny M, Bleeker W (2004) Comparative ITS and AFLP analysis of diploid *Cardamine* (Brassicaceae) taxa from closely related polyploid complexes. Annals of Botany, 93, 507-520.

Mariette S, Le Corre V, Austerlitz F, Kremer A (2002) Sampling within the genome for measuring within-population diversity: trade-offs between markers. Molecular Ecology, 11, 1145-156.

Marshall ES (1892) On *Cochlearia Groenlandica* L. Journal of Botany, 30, 225-226.

Marshall ES (1893) *Cochlearia* Sp. Groves, J., Botanical Exchange Club London, James Collins & Co, London

Marshall ES (1894) On an apparently undescribed *Cochlearia* from Scotland. Journal of Botany, 32, 289-292.

Martínez-Ortega M, Montserrat DL, Albach, DC, Elena -Rosselló, JA, Rico E (2004) Species boundaries and phylogeographic patterns in cryptic taxa inferred from AFLP markers: *Veronica* subgen. *Pentasepalae* (Scrophulariaceae) in the Western Mediterranean. Systematic Botany, 29, 965-986.

Mayr E (1942) Systematics and the origin of species. Columbia University Press, New York.

Mayr E (1963) Animal species and evolution. Harvard University Press, Cambridge, Massachusetts.

Meudt HM, Clarke AC (2007) Almost forgotten or latest practice? AFLP applications, analyses and advances. Trends in Plant Science, 12, 106-117.

Mishler BD, Donoghue MJ (1982) species concepts: a case for pluralism. Systematic Zoology 31, 491-503.

Moreau M, Laffly D, Joly D, Brossard T (2005) Analysis of plant colonisation on an arctic moraine since the end of the little ice age using remotely sensed data and a Bayesian approach. Remote Sensing of the Environment, 99, 244-253.

Moritz C (2002) Strategies to protect biological diversity and the evolutionary processes that sustain it. Systematic botany, 51, 238-254.

Morjan CL, Rieseberg LH (2004) How species evolve collectively: implications of gene flow and selection for the spread of advantageous alleles. Molecular Ecology, 13, 1341-1356.

Mueller UG, Wolfenbarger LL (1999). AFLP genotyping and fingerprinting. Trends in Ecology and Evolution 14, 389-394.

Nagy L, Proctor J (1997) Plant growth and reproduction on a toxic alpine ultramafic soil: adaptation to nutrient limitation. The New Phytologist, 137, 267-275.

Nixon KC, Wheeler QD (1990) An amplification of the phylogenetic species concept. Cladistics, 6, 211-223.

Nordal I (1988) *Cochlearia pyrenaica* DC., a species new to Scotland. Watsonia, 17, 49-52.

Nordal I, Eriksen AB, Laane MM, Solberg Y. (1986) Biogeographic and biosystematic studies in the genus *Cochlearia* in Northern Scandinavia. Symbolae Botanicae Upsaliensis, 27, 83-93.

Nordal I, Laane MM (1990) Cytology and Reproduction in arctic *Cochlearia.* Sommerfeltia, 11, 147-158

Nordal I, Laane MM (1996) Taxonomic delimitation within the *Cochlearia* officinalis s. lat. with particular discussion on the rank of *C. anglica* (Brassicaceae). Symbolae Botanicae Upsaliensis, 31, pp. 47-57

Nordal I, Stabbetorp OE (1990) Morphology and taxonomy of the genus *Cochlearia* (Brassicaceae) in Northern Scandinavia. Nordic Journal of Botany, 10, 249-263.

Odasz AM (1994) Nitrogen reductase activity in the vegetation below an arctic bird cliff, Svalbard, Norway. Journal of Vegetation Science, 5, 913-920.

Özkan H, Brandolini A, Schäfer-Pregl R, Salamini F (2002) AFLP analysis of a collection of tetraploid wheats indicates the origin of Emmer and Hard Wheat domestication in Southeast Turkey. Molecular Biology and Evolution, 19, 1797-1801.

Paschke M, Clemens A, Schmid B (2002) Relationship between the population size, allozyme variation, and plant performance in the narrow endemic *Cochlearia bavarica*. Conservation Genetics, 3, 131-144.

Paun O, Greilhuber J, Temsch EM, Horandl E (2006) Patterns, sources and ecological implications of clonal diversity in apomictic *Ranunculus carpaticola* (*Ranunculus auricomus* complex, Ranunculaceae). Molecular Ecology, 15, 897-910.

Pegtel DM (1999) Effect of ploidy level on fruit morphology, seed germination and juvenile growth in scurvy grass (*Cochlearia officinalis* L. s.l., Brassicaceae). Plant Species Biology, 14, 201-215.

Pillon Y, Qamaruz-Zaman F, Fay MF, Hendoux F, Piquot Y (2007) Genetic diversity and ecological differentiation in the endangered fen orchid (*Liparis loeselii*). Conservation Genetics, 8, 177-184.

Pobedimova E (1969) Revisio generis *Cochlearia* L., 1 Novosti sistematiky Vysshikh Rastenii. Leningrad, 6, 67-106.

Pobedimova E (1970) Revisio generis *Cochlearia* L., 2 Novosti sistematiky Vysshikh Rastenii. Leningrad, 7, 167-195.

Preston CD, Pearman DA, Dines TD (2002) New Atlas of British Flora. Oxford University Press, Oxford.

Qiagen (2006) DNeasy™ Plant Mini Kit and DNeasy Plant Maxi Kit Handbook, for DNA isolation from plant tissue. QIAGEN, Inc.

Reid EM (1949) The late glacial flora of the Lea valley. New Phytologist, 48, 245-252.

Rich TCG, Dalby DH (1996). The Status and Distribution of Mountain Scurvygrass (*Cochlearia micacea* Marshall) in Scotland with ecological notes. Botanical Journal of Scotland, 48, 187-198.

Rieseberg LH (1995) The role of hybridization in evolution: old wines in new skins. American Journal of Botany, 82, 944-953.

Rieseberg LH, Soltis DE (1991) Phylogenetic consequences of cytoplasmic gene flow in plants. Evolutionary Trends in Plants, 5, 65-83.

Robertson A, Newton AC, Ennos RA (2004) Multiple hybrid origins, genetic diversity and population genetic structure of two endemic *Sorbus taxa* on the Isle of Arran, Scotland. Molecular Ecology, 13, 123-134.

Rouppe van der Voort JNAM, Van Zandvoort P, Van Eck HJ, Folkertsma RT, Hutten RCB, Draaistra J, Gommers FJ, Jacobsen E, Helder J, Bakker J (1997) Use of allele specificity of comigrating AFLP markers to align genetic maps from different potato genotypes. Molecular genomics and genetics, 255, 438-477.

Rozema J, Bijwaard P, Prast G, Broekman R (1985) Ecophysiological adaptations of coastal halophytes from foredunes and saltmarshes. Plant Ecology, 62, 499-521.

Russell RS, Ward Cutler D, Jacobs SE, King A, Pollard AG (1940) Physiological and ecological studies on an arctic vegetation: II The development of vegetation in relation to nitrogen supply and soil micro organisms on Jan Mayen Island. Journal of ecology, 28, 269-288.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning a Laboratory Manval, 2nd ed. Cold Spring Harbor Laboratory Press, New York.

Saunté LH (1955) Cyto-genetical studies in the *Cochlearia officinalis* complex. Hereditas, 41, 500-515.

Schaal BA, Hayworth DA, Olsen KM, Rauscher JT, Smith WA (1998) Phylogenetic studies in plants: problems and prospects. Molecular Ecology, 7, 465-474.

Scheen A-C, Elven R, Brochmann C (2002) A molecular-morphological approach solves taxonomic controversy in arctic *Draba* (Brassicaceae). Canadian Journal of Botany, 80, 59-71.

Schmidt K, Jensen K (2000) Genetic structure and AFLP variation of remnant populations in the rare plant *Pedicularis palustris* (Scrophulariaceae) and its relation to population size and reproductive components. American Journal of Botany, 87, 678-689.

Schmidt-Lebuhn AN (2007) Using amplified fragment length polymorphisms to unravel species relationships and delimitations in *Minthostachys* (Labiatae). Botanical Journal of the Linnean Society, 153, 9-19.

Schneider S, Roessli D, Excoffier L (2006) Arlequin version 3.1: a software for population genetic data analysis. University of Geneva.

Schönswetter P, Popp M, Brochmann C (2006a) Central Asian origin of and strong genetic differentiation among populations of the rare and disjunct *Carex atrofusca* (Cyperaceae) in the Alps. Journal of Biogeography, 33, 948-956.

Schönswetter P, Popp M, Brochmann C (2006b) Rare arctic-alpine plants of the european alps have different immigration histories: the snow bed species *Minuartia biflora* and *Ranunculus pygmaeus*. Molecular Ecology, 15, 709-720.

Schönswetter P, Tribsch A, Niklfeld H (2004) Amplified fragment length polymorphisms (AFLP) suggests old and recent immigration into the Alps by the arctic-alpine annual *Comastoma tenellum* (Gentianaceae). Journal of Biogeography, 31, 1673-1681.

Schönswetter P, Tribsch A, Schneeweiss GM, Niklfeld H (2003) Disjunction in relict alpine plants: phylogeography of *Androsace brevis* and *A. wulfeniana* (Primulaceae). Botantical Journal of the Linnean Society, 141, 437-446.

Scott N (1985) The updated distribution of maritime species on British roadsides. Watsonia, 15, 381-396.

Sharbel TF, Haubold B, Mitchell-Olds T (2000) Genetic isolation by distance in *Arabidopsis thaliana*: biogeography and postglacial colonization of Europe. Molecular Ecology, 9, 2109-2118.

Sharbel TF, Mitchell-Olds T (2001) Recurrent polyploid origins and chloroplast phylogeography in the *Arabis holboellii* complex (Brassicaceae). Heredity, 87, 59-68.

Shull G (1918) The duplication of the leaf-lobe factor in Shepherd's Purse. Brooklyn Botanic Garden Memoirs, 1, 427-443.

Shull G (1929) Species hybridisation among old new species of Shepherds purse. The Proceedings of the International Congress of Plant Science, 1, 837-888.

Soltis DE, Soltis PS (1993) Molecular data and the dynamic nature of polyploidy. Critical Reviews in Plant Science, 12, 243-273.

Soltis DE, Soltis PS (1995) The dynamic nature of polyploid genomes. Proceedings of the National Academy of Sciences of the USA, 92, 8089-8091.

Soltis DE, Soltis PS, Tate JA (2004) Advances in the study of polyploidy since *Plant speciation.* New Phytologist, 161, 173-191.

Song K, Lu P, Tang K, Osborn TC (1995) Rapid genome change in synthetic polyploids of *Brassica* and its implications for polyploid evolution. Proceedings of the National Academy of Sciences of the USA, 92, 7719-7723.

Spieth PT (1974) Gene flow and genetic differentiation. Genetics, 78, 961-965.

Squirrel J, Hollingsworth PM, Bateman RM, Tebbit MC, Hollingsworth ML (2002) Taxonomic complexity and breeding system transitions: conservation genetics of the *Epipactis leptochila* complex (Orchidaceae). Molecular Ecology, 11, 1957-1964.

Stace CA, (1975) Hybridisation and the Flora of the British Isles. The Botanical Society of the British Isles. Academic Press, London.

Stace C (1997) New Flora of the British Isles. Cambridge, Cambridge University Press.

Stanton ML, Galen C (1997) Life on the edge: adaptation versus environmentally mediated gene flow in the snow buttercup, *Ranunculus adoneus*. The American Naturalist, 150, 143-178.

Tero N, Aspi J, Silkamaki P, Jakalaniem A, Tuomi J (2003) Genetic structure and gene flow in a metapopulation of an endangered plant species, *Silene tatarica*. Molecular Ecology, 12, 2073-2085.

UK biodiversity steering group (1995) Species action plan for *Cochlearia micacea* (1995) Biodiversity: The UK Steering Group Report Action Plans HMSO, London Volume II pp. 177.

Vekemans X, Beauwens T, Lemaire M, Roldan Ruiz I (2002) Data from amplified fragment length polymorphism (AFLP) markers show evidence of size homoplasy and of a relationship between the degree of homoplasy and fragment size. Molecular Ecology, 11, 139-151.

Vijverberg K, Kuperus P, Breeuwer JAJ, Bachmann K (2000) Incipient adaptive radiation of New Zealand and Australian *Microseris* (Asteraceae): an amplified fragment length polymorphism (AFLP) study. Journal of Evolutionary Biology, 13, 997-1008.

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research, 23, 4407-4414.

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. Evolution, 38, 1358-1370.

Wendt T, Canela MBF, Klein DE, Rios RI (2002) Selfing facilitates reproductive isolation among three sympatric species of *Pitcairnia* (Bromeliaceae). Plant Systematics and Evolution 232, 201-212.

West RG, Dickson CA, Catt JA, Weir AH, Sparks BW (1974) Late Pleistocene deposits at Wretton, Norfolk II. Devensian Deposits. Philosophical Transactions of the Royal Society of London, Series B Biological Sciences, 267, 337-420.

Wilson EO, Brown WL (1953) The subspecies concept and its taxonomic application. Systematic Zoology, 2, 97-111.

Winfield MO, Wilson PJ, Labra M, Parker JS (2003) A brief evolutionary excursion comes to an end: the genetic relationship of British species of *Gentianella* sect. *Gentianella* (Gentianaceae). Plant Systematics and Evolution, 237, 137-151.

Woodell SRJ, Dale A (1993) *Armeria maritima* (Mill.) Willd. (*Statice armeria* L.; *S. maritima* Mill.). The Journal of Ecology, 81, 573-588.

Wyse Jackson PS (1991) A note on *Cochlearia scotica* Druce (Crucifereae). Flora Europeae: Notulae systematicae ad floram Europaeam spectantes series 2 / 4, 106, 97-119.

Zhang L-B, Comes HP, Kadereit JW (2001) Phylogeny and quarternary history of the European montane/alpine endemic *Soldanella* (Primulaceae) based on ITS and AFLP variation. American Journal of Botany, 88, 2331-2345.

Zhivotovsky LA (1999) Estimating population structure in diploids with multilocus dominant DNA markers. Molecular Ecology, 8, 907-913.